PB-230 335

RELATIVE TOXICITY OF ORGANICS TO 'CHLORELLA PYRENOIDOSA'

D. Thirumurthi, et al

Texas University

Prepared for:

Federal Water Pollution Control Administration

1 November 1965

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The University of Texas

CENTER FOR RESEARCH IN WATER RESOURCES

Environmental Health Engineering Research Laboratory

Department of Civil Engineering

RELATIVE TOXICITY OF ORGANICS TO CHLORELLA PYRENOIDOSA

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U. S. Public Health Service
Water Supply and Pollution Control Division
Contract WP-00688-01A1

November 1, 1965

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ACKNOWLEDGMENT

This research was made possible in part through U.S. Public Health Service Water Supply and Pollution Control Division Grant WP-00688-01A1 Effect of Toxic Organics on Waste Stabilization.

Special acknowledgments are extended to faculty and staff of The University of Texas for their guidance. Particular appreciation is due to Dr. Jack Myers and Miss J.R. Graham of the Algal Physiology Laboratory. Dr. Orville Wyss, Chairman of the Department of Microbiology, and Civil Engineering faculty members Dr. W.L. Moore, Dr. J.F. Malina, and Dr. J.O. Ledbetter assisted as faculty advisers.

ACKNOWLEDGMENT

The author expresses his appreciation and gratitude to all those who have made the completion of this dissertation possible. This study was carried out under the supervision of Dr. Earnest F. Gloyna. The author is deeply indebted to him for his guidance and interest.

Special acknowledgments are extended to faculty and staff of The University of Texas for their guidance. Particular appreciation is due to Dr. Jack Myers and Miss J.R. Graham of the Algal Physiology Laboratory. Dr. Orville Wyss, Chairman of the Department of Microbiology, and Civil Engineering faculty members Dr. W.L. Moore, Dr. J.F. Malina, and Dr. J.O. Ledbetter assisted as faculty advisers.

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Special appreciation is due to the author's wife for her understanding and support.

RELATIVE TOXICITY OF ORGANICS TO

CHLORELLA PYRENOIDOSA

Publication No. _____

Dhandapani Thirumurthi, Ph.D.
The University of Texas, 1966

Supervising Professor: Earnest F. Gloyna

The relative toxicity of certain short chain fatty acids, dicarboxylic acids, alcohols, aldehydes and some selected organic compounds to Chlorella pyrenoidosa were evaluated. Changes in chlorophyll content of the algal suspension were measured to determine toxicity. In addition, studies were conducted to investigate the effect of certain inactivators in reducing the toxicity of some of the organic chemicals.

The environmental factors were uniformly controlled. The nutrient supply consisted of a well-established inorganic medium and a gas supply containing five per cent by volume of carbon dioxide in air. The test facility consisted of a special tank containing a fluorescent light source, a gas manifold and a water bath.

The results show that modifications to industrial waste treatment plant design practices are needed, since decreases in stream reaeration rates may be significant if certain chemical wastes are discharged. A general relationship exists between toxicity and molecular structure of the chemicals investigated. The toxicity of some chemicals can be neutralized by the addition of certain metabolic intermediates. However, the latter is not suggested as a design consideration.

CONCLUSIONS

1. The design standards of waste stabilization ponds can be improved in accordance with the toxicity values determined in this investigation. Certain organic materials, if present in sufficient concentrations, reduce the chlorophyll production, thereby decreasing the photosynthetic capacity of the algal cells. Toxicity correction coefficients should be included in the presently used design formulas.

2. A general relationship exists between the toxicity and molecular structure, i.e., structurally similar groups of fatty acids, dicarboxylic acids, alcohols, and aldehydes.

a. The short chain fatty acids with odd number of carbon atoms in the molecules are more toxic to Chlorella pyrenoidosa than the corresponding acids with even number of carbon atoms. The toxic effects of primary alcohols increase with increasing numbers of carbon atoms. In the case of dicarboxylic acids, the toxicity decreases to a minimum and then increases with increasing numbers of carbon atoms.

b. Butanoic and pentanoic acids, straight chain fatty acids, are more toxic than the corresponding branched chain fatty acids. The straight chain aldehyde, 1-butanal reduces the chlorophyll content of the algae more than the corresponding branched chain aldehyde. Similarly, the primary alcohols 1-propanol, 1-butanol, and 1-hexanol are more toxic than the corresponding secondary or tertiary alcohols, 2-propanol, 3-butanol, and 2-hexanol. However, as an exception to the relationship, 1-butanol is not more toxic than 2-butanol.

3. The toxic effects of some compounds can be minimized by the addition of certain inactivators.

a. The toxic effects of 2-thiouracil are neutralized by the structurally similar compound, uracil. The compound 2-thiouracil is toxic, probably as a result of competitive inhibition. Also, the presence of adenine neutralizes the toxic effects of 2-thiouracil. However, the presence of orotic acid, cytidylic acid, and hypoxanthine does not show any evidence of inactivating the toxic effects of 2-thiouracil.

b. The toxic effects of ethanol are neutralized by phosphoglyceric acid (PGA). Hence, it seems that the toxicity of ethanol to algae results from a suppression of metabolic intermediates such as PGA. However, this conclusion cannot be extended to the other alcohols. The toxic effects of methanol and 1-propanol are not neutralized in the presence of PGA.

c. An excessive concentration of iron and sulfur (500 to 2000 mg/L of ferrous sulfate) neutralizes the toxicity of the chelating agent, o-phenanthroline, whereas PGA is ineffective. Hence, it may be suggested that o-phenanthroline is toxic to algae because the chelating agent forms an iron complex with the reducing enzyme, pyridine nucleotide reductase, thereby inactivating the enzyme. However, in the presence of excessive iron, the chelating agent forms an iron complex with the excess iron added in the form of ferrous sulfate leaving the iron in the enzyme unaffected. Hence, the reducing enzyme is available to enter into the metabolic processes.

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CHAPTER I

INTRODUCTION

As industrial production increases, there will be a corresponding increase in the discharge of wastewaters containing toxic organic compounds. These toxic chemicals, if present in sufficient concentrations, may effectively eliminate the biological life in streams, lakes, estuaries and waste stabilization ponds. Trace amounts of certain non-biodegradable compounds may accumulate and eventually interfere with stream reaeration processes by destroying the green algae or at least limit the photosynthetic processes.

Objectives

The purpose of this research was to determine the relative toxicity of certain organic compounds to a culture of Chlorella pyrenoidosa. Specifically, the objective was to suggest design modifications for waste stabilization ponds in conjunction with the toxicity values.

In this connection it was necessary to relate the toxic effects of selected materials with their molecular structure. A further aim was to investigate the capacities of certain inactivators in reducing the toxicity of some of the organic compounds.

Scope

Chlorella pyrenoidosa was the test organism. Precautions were taken to eliminate variations in the test organism. Also,

measures were undertaken to prevent gross bacterial contamination.

The toxic effects of the pollutants on the algae were evaluated by measuring changes in the concentration of chlorophyll. This measurement proved to be more precise than the turbidimetric or gravimetric measurements commonly used to estimate cell concentrations.

Selected compounds consisted of organic acids, alcohols, aldehydes and certain other organic chemicals. The criteria for selection was based largely upon solubility and volatility. Initially it was deemed necessary to select compounds which were soluble and relatively non-volatile.

Environmental control was rigidly established. The initial pH, algal mass and nutrients were fixed. The temperature was controlled between 25°C to 30°C. An excess available carbon source was supplied as carbon dioxide. A five per cent carbon dioxide-air mixture was used to maintain an excess of available carbon and to keep the algal cells in suspension. The release of carbon dioxide and agitation was uniformly controlled.

The duration of a test period was five days. This time interval was selected on the basis of past experience. A longer test period was not practical because of the general depletion of inorganic nutrients. Yet, the test was sufficiently long to effectively demonstrate major changes in the chlorophyll content.

CHAPTER II

LITERATURE REVIEW

In recent years much research has been directed toward the treatment of industrial wastes which contain toxic materials. This chapter presents an evaluation of the literature as related to a general definition of toxicity, toxic effects of organics on algae and bacteria, inactivation of the toxic effects in higher plants, and characteristics of algae.

Definition of Toxicity

It must be emphasized that the methods for toxicological testing, definitions and interpretations of the results have not been standardized. However, individuality and originality may be used to arrive at a solution of a practical problem. According to Gerarde (16), toxicology is more than the routine testing of chemicals; it is biological research designed to find out as quickly as possible the toxic effects of a chemical on a given living organism.

The most important part of a toxicity study is the statistical interpretation of the experimental data. This evaluation is derived by experience and judgment coupled with sound training in the biological and chemical sciences. Consequently, the degree of toxicity may be related to the chemical structure, molecular weight or some other chemical parameter (16).

Stokinger (49) has classified the toxicological problems in water pollution into three major areas; namely, chemical synthetics, accumulation of solids, and trace substances. In the first area, the chief reasons for concern are their increasing

number, their structural complexity, and faulty metabolic behavior of synthetic organic compounds. Stokinger feels that, in addition to the challenge of identification, many of these toxic pollutants exist in wastewater in low concentrations and the problem is more complicated due to the side reaction products accompanying manufacture and the products of biological degradation.

George (15) draws attention to the necessity of fundamental research which may lead to the new development of basic principles, biochemical-toxicological laws and possibly short-cut methods that will finally bring down the cost of experimental procedures. While the possibility of discovering a new law or formula that would express universal or even broad application seems remote, it should be possible at least to develop consistent trends that might relate toxicity to specific groups or compounds.

When the toxicity of a chemical is defined with respect to a given organism, the environmental factors must be clearly expressed. Fitzgerald, Gerloff and Skoog (11) showed that 2,3-dichloronaphthoquinone was an inhibitor of blue-green algae when applied at concentrations from two to ten µg/L (micrograms per liter). However, under continued alkaline conditions this chemical was hydrolyzed to 2-chloro-3-hydroxy naphthoquinone. This new compound was not an effective algicide and was toxic at a concentration of one to two mg/L.

Fitzgerald and Faust (9) have defined the algicidal and algistatic properties of a chemical. Whether a toxic level

of a chemical is algicidal or algistatic is determined by removing a sample of algal suspension from the environment of the toxic chemical and placing it in a suitable culture medium. If the sub-cultures of the algae do not revive, the concentration of the chemical is considered as algicidal. If the algae start to grow under appropriate incubation, the concentration is said to be algistatic.

Experts on the subject of toxicology are divided in their opinions regarding the evaluation of data (16). Some of them prefer to evaluate toxicity by tests on living organisms and others prefer to measure specific toxic substances by chemical analysis. Toxicity of organic chemicals to aquatic life can be evaluated to some extent in the laboratory by means of aquarium tests (6). The vital factors to be controlled are selection of test organism with respect to species, acclimatization and physical fitness of the specimens, number of specimens, duration of test, feeding habits during the test, sources of water supply, temperature, pH, liquid depth and aeration. Renn (43) noticed that hardness, total alkalinity and pH can greatly influence the toxicity of cyanide wastes. The toxicity was found to be less when hard water was used as compared to soft water.

Toxic Effects of Organics on Algae

Lefrancois and Quellet (28) have conducted experiments on the photosynthetic assimilation of $C^{14}O_2$ by Scenedesmus in the presence of ethanol and o-phenanthroline. It was found that the fixation of $C^{14}O_2$ into some key compounds such as phosphoglyceric

acid was suppressed during these tests, while the rate of fixation into other compounds such as glycolic acid and the simple amino acids remained nearly normal or was accelerated. These experiments have also shown that the inhibiting power of the straight chain alcohols increased correspondingly with the number of carbon atoms in the molecule.

Several kinds of α-hydroxysulfonates, the bisulfite addition compounds of aldehydes and ketones, were found to be inhibiting the photosynthetic carbon dioxide fixation of Chlorella cells as indicated by Asada and Kasai (4). Bisulfite addition compounds of glyoxal, glyoxylate and benzaldehyde were more effective in their toxicity than those of formaldehyde and acetaldehyde.

In photosynthesis, the locus of participation of the reducing agent (or photogenic agent) is assumed to be the step when phosphoglyceric acid is reduced to triose phosphate. Experimenting with Chlorella, Miyachi (37) has suggested that the site of action of iodoacetamide, which has been known to be a strong inhibitor of photosynthesis, is the site of photochemical formation of the reducing agent from its precursor.

Seventeen different toxicants have been tested by Ravenna (42) against the growth of five species of algae which included Monochrysis lutheri, Dunaliella euchlora, Chlorella sp., Protococcus sp., and Phaeodactylum tricornutum. The chemicals tested included organic phosphates, phenol, chlorinated hydrocarbons, and substituted urea compounds. The toxicity rating was found to be: Diuron > Monuron > Lignasan > DDT > Toxaphene > Lindane >

chloronitropropane > orthodichlorobenzene > Dowacide A > phenol > Diptrex.

Two chlorinated compounds, dodecyl guanidine hydrochloride and trimethylalkyl ammonium chloride at concentrations of two mg/L were algicidal to all the blue-green algae and diatoms tested and were algistatic to all but two species of green algae. The compound 1,3,4,6 tetrachloroglycouril at the same concentration was algicidal to all species of green algae and diatoms tested (1).

The control of blue-green algal blooms with 2,3-dichloronaphthoquinone (2,3-DNQ) in a ten ft deep lake has been investigated by Fitzgerald and Skoog (12). From 20 to 53 parts per billion of 2,3-DNQ was found effective in eliminating even very heavy blooms of blue-green algae.

Maloney and Palmer (32) have surveyed the toxicity of six chemical compounds to 30 cultures of algae. The six chemicals were copper sulfate, 2,3-DNQ, dodecylacetamidodimethyl benzyl ammonium chloride (DAC), rosin amine D acetate (RADA), zinc dimethyl dithio carbonate (ZDD) and rosin amine D sulfate (RADS). At 0.5 mg/L, 2,3-DNQ destroyed 28 per cent of the blue-green algae without being toxic to any of the diatoms and green algae. The toxicity rating was found to be: ZDD > RADA > DAC > RADS > copper sulfate > 2,3-DNQ.

The algicidal properties of certain natural products were evaluated by Safferman and Morris (44). The extracellular materials of 545 actinomycete and fungal isolates showed a wide distribution of agents having algicidal activities.

Inhibition of photosynthesis in Rhodopseudomonas palustris by the reduced forms of dyes such as methylene blue, brilliant cresyl blue and toluidine blue have been reported by Izawa (25). It is suggested that these dyes may act in one of two possible ways and inhibit the photosynthetic metabolism. According to the author, Izawa, these dyes may act as oxidation reduction catalysts and form a short circuit in the photosynthetic process. Thus the flow of electrons for the reduction of carbon dioxide is blocked. Another possibility is that the reduced forms of these dyes may affect photosynthesis by merely reducing some essential components in the metabolism. The author indicates that the former possibility is more probable, although the latter one cannot be disregarded completely.

Algicidal properties of Armazide, a swimming pool additive, were evaluated by Antonides and Tanner (2). This chemical was effective in controlling the growth of algae at a concentration of 4.5 mg/L. It was suggested that the hardness encountered in the natural waters of different geographic areas should be considered while determining the dose of Armazide in swimming pools.

Korzep (27) tested some organic compounds and one petrochemical waste for their toxic effects on Chlorella pyrenoidosa. He found an increasing order of toxicity for organic acids, alcohols and amines, alkyl halides, aldehydes and ketones, and benzene series compounds.

Toxic Effects of Organics on Bacteria

Scott and Wolf (45) have discussed the anti-bacterial activity of a series of quaternaries prepared from hexa methylene tetramine

(HMN) and halo hydrocarbons. The compounds with the lower toxic effect had a nitrogen bonded to a carbon atom in the α position.

Gershon and Parmegiani (18) conducted experiments involving the anti-bacterial activity of ring poly-chlorinated pyrimidines. It was reported that the addition of a side chain to the ring generally reduced the anti-microbial activity and the reduction in activity was proportional to the size of the side chain.

The anti-microbial activities of 8-quinolinol, its salts with salicylic acid and 3-hydroxy-2-naphthoic acid, and the respective copper chelates in liquid have been investigated by Gershon, et al. (17). In liquid culture, the salts of 8-quinolinol were not found to be as active against bacteria as 8-quinolinol alone.

Fitzgerald, et al. (11) have suggested a general relationship between toxicity and chemical structure of the quinone type compounds. Any compound with two hydrogen atoms which can be oxidized from -OH or $-NH_2$ groups which are ortho or para to each other or which bear some similar relationship, will give rise to a fairly stable oxidant and will be toxic. The structure of the rings and the additions to the rings merely changed the effects of the redox character and thus the toxicity. Ortho and para directing groups usually weaken the bond holding the hydrogen atom to the oxygen or nitrogen and tend to increase the toxicity. Whereas, meta directing groups weaken the hydrogen bonding to oxygen or nitrogen and tend to decrease the toxicity. The above principles were derived by conducting experiments with pure cultures of Microcystis aeruginosa.

Inactivation of Toxic Effects in Higher Plants

The growth inhibiting effects of 5-bromouracil (5-BMU) and 5-fluorouracil (5-FU) on the germination of Eragrostis seed has been studied by Tadashi (50). It has been found that the seedling growth was effectively inhibited by 5-FU and the inhibition was reversed by uracil, uridine and orotic acid but not by thymine and thymidine. The author suggested that the inhibitory effect of 5-FU may be due to the depressing action of 5-FU upon RNA synthesis.

Shimeno and Shukuo (46) have examined the effects of certain nucleic acid anti-metabolites upon the germination of leaf mustard seed. The authors indicated that among the anti-metabolites tested, only 2-thiouracil showed a strong inhibitory action upon the germination. This inhibition by 2-thiouracil was relieved markedly by such pyrimidine derivatives as uracil, thymine, orotic acid, uridylic acid and cytidylic acid. On the other hand, such a relieving effect was not shown by purine derivatives such as adenine, hypoxanthine and xanthine. The authors suggested that 2-thiouracil may compete in the plant with uracil as reported by several other investigators (26, 34 and 35).

According to Gray and Hendlin (21), chlorophyll formation was greatly inhibited in such higher plants as bean and wheat by DL-o-methylthreonine. The amino acid analog, DL-o-methylthreonine, was applied as a spray at a concentration of 1000 mg/L to bean and wheat plants. The new leaves which grew after the spray treatment were completely bleached. The chlorophyll formation was inhibited. However, addition of four to nine times as much

DL-isoleucine as DL-o-methylthreonine neutralized the inhibiting effects of o-methylthreonine. Chlorophyll formation was inhibited also in Euglena gracilis by 100 mg/L of o-methylthreonine in the growth medium. The inhibition was completely reversed by adding ten times as much DL-isoleucine. DL-leucine and DL-norleucine also showed similar reversing effects.

The growth modifying and antimetabolite effects of certain amino acids on chrysanthemum plants were described by Woltz (52). Four of the natural amino acids (L-isoleucine, L-leucine, L-methionine and L-valine) and seven synthetic amino acids (DL-alloiso leucine, DL-norleucine, DL-ethionine, DL-methionine, DL-valine, DL-isovaline and DL-norvaline) exhibited significant growth modifying effects. Proceeding on the hypothesis that many of the inhibiting effects might have been due to antimetabolites, amino acids or their analogues were applied with offending amino acids. In practically all such cases, growth was improved and the adverse effects prevented or reduced in severity.

The inhibitory effects on carbon dioxide fixation, ATP formation and TPN reduction by spinach chloroplasts have been discussed by Bamberger et al. (5). The three compounds which inhibited TPN reduction and the associated phosphorylation were 3-(p-chlorophenyl)-1, 1-dimethylurea (CMU), 2-heptyl-4-hydroxyquinoline-N-oxide and 2-nonyl-4-hydroxyquinoline-N-oxide. The inhibitory effect could be overcome by the addition of 2, 6-dichlorophenol indophenol (DCIP) and ascorbate. m-Chlorocarbonyl cyanide phenylhydrazone (CCCP) was a potent uncoupler of phosphorylation. It was noted that CCCP inhibited carbon dioxide fixation but this

effect could be overcome by the addition of fructose-1, 6 diphosphate.

The effects of the iron chelating agent o-phenanthroline on the photosynthetic enzyme, pyridine nucleotide reductase, were investigated by Fry and Pietrs (14). It was found that if the enzyme was incubated at about 80°F for five minutes at a pH of five with o-phenanthroline, 97 to 100 per cent of the iron was released as ferrous triphenanthrolate complex. The data indicated that the addition of o-phenanthroline resulted in a loss of iron and labile sulfur from the enzyme. These changes in iron and sulfur content resulted in the inability of the enzyme to reduce triphosphopyridine nucleotide.

Characteristics of Algae

The test organism selected for this study was the green alga, Chlorella pyrenoidosa. Some description of the characteristics of the test algae are presented below. The growth characteristics of other algal species in toxic environments are also discussed.

Myers (39) has indicated that Chlorella has become a "standard" organism in algal research. The Emerson strain of Chlorella, namely, Chlorella pyrenoidosa, has long been recognized as the most widely used species in laboratory work. The cells are approximately spherical in shape and three to eight microns in diameter. The optimum conditions for best growth of the test algae are: temperature control at 25°to 30°C, inorganic nutrients, a carbon source with five per cent carbon dioxide and a light source of about 500 foot candles (27).

Similar to the growth habits of other organisms, algae can adjust to environmental changes and can develop a degree of resistance to certain toxic chemicals. However, Fitzgerald (8) points out that care should be taken so as not to claim the development of resistance to a chemical. Actually, some other factors in the environment may be responsible for the apparent resistance.

Certain species of algae can develop special resistance and grow when toxic chemicals are introduced into the environment. The blue-green alga, Anabaena cylindrica, can secrete into the medium certain substances and thereby grow under some adverse conditions. Fogg and Westlake (13) showed that in the presence of extracellular polypeptides the toxicity of copper decreased. These polypeptides may be secreted into the culture medium by Anabaena cylindrica.

The protection against toxic chemicals can be obtained from the sheath material surrounding algal colonies or from the outer cells of dense mats of algae. For example, the blue-green algae, Phormidium retzii, can be enclosed in a protective layer of mucilage and grown in several compressed layers (9).

The stability and reversibility of adaptation to selenomethionine by Chlorella vulgaris has been discussed by Shrift et al. (47). The algal cells developed resistance to the growth uncoupling effects of selenomethionine. This resistance was manifested by the Chlorella cells even after the removal of the amino acid analogue from the environment. The developed resistance was reversed when the adapted cells were subjected to sulfur

starvation or when the cells were provided with D-methionine as the only sulfur source.

CHAPTER III

EQUIPMENT AND PROCEDURE

The development of this research required the construction of a specially designed test apparatus and the use of a generally accepted chlorophyll extraction procedure.

Equipment

The test chamber consisted of an aquarium complete with a light source, a gas manifold and an apparatus for holding test tubes. The aquarium was constructed of Lucite as shown in Fig. 3-1. The thickness of the walls and bottom, respectively, were 0.25 and 0.5 inches. To hold the test tubes which were used as the actual test vessels, a fiber board with spaced holes was positioned near the top of the aquarium. Tap water was circulated through the aquarium to maintain relatively constant temperature between 25 and 30°C.

Four fluorescent lamps of 200 watts each with attached aluminum reflectors were used to provide a continuous light source. Two lamps were positioned on each side of the aquarium. Each lamp was 48 inches in length.

The gas manifold was constructed in such a manner that the air-carbon dioxide mixture could be piped to any one of 60 outlets. A master regulator valve was used to reduce the tank pressure to a usable level. Attached to the central manifold were the various outlets. The flow through each outlet could be controlled with a needle valve.

Flexible rubber hoses were used to convey the gas mixture from the needle valves into the test tubes. Capillary glass

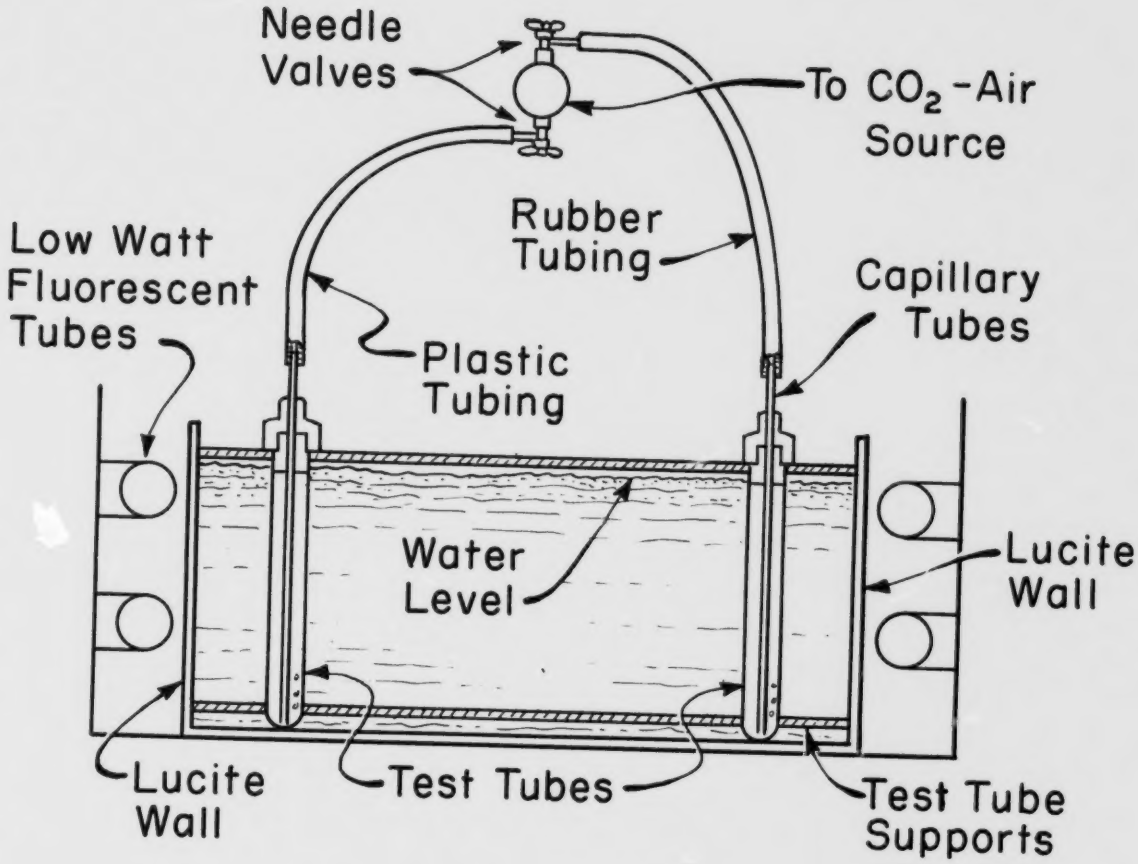

FIG. 3-1. TEST APPARATUS

tubes were connected to each rubber hose and of such length as to extend to the bottom of the test tubes. Each capillary glass tube was inserted through a rubber bulb of an eye dropper bottle cap. This cap provided a cover for the top of the test tube and thereby prevented gross air-borne contamination.

A Beckman Spectrophotometer, Model B, was used for all chlorophyll analysis. This spectrophotometer is a single beam instrument providing rapid transmittance measurements in the 320 to 1000 mμ range.

pH measurements were made with a Beckman pH meter, Model/H2. Standardizations were made with phosphate and acetate buffer solutions.

An International Clinical Centrifuge was used during the chlorophyll extraction procedures. Maximum speed attainable was 3400 rpm. This maximum speed provided a centrifugal force of 1550 dynes (gm-cm/ sec^2).

A Brahmall-Deane autoclave was used to sterilize the test tubes, aluminum caps, glassware, rubber hose, and capillary glass tubes prior to each test. The autoclave was operated at 250°F with a pressure of 15 p.s.i. for 15 minutes.

Experimental Procedure

The experimental procedure involved preliminary tests, final toxicity tests and neutralization studies. The effect of pH and volatile characteristics of the organic chemicals on algal growth were also investigated. An inactivation study was undertaken to determine the neutralization of the toxic characteristics of certain chemicals to algae through the utilization of metabolic

intermediates.

Test Procedure

During the preliminary tests, concentrations varied between one and 50,000 mg/L. The actual range depended on the specific chemical. These preliminary tests were very helpful in establishing toxicity ranges. About 10 to 15 test tubes were used during each preliminary test and about 25 to 35 tubes were used during the final test.

A typical test tube contained five ml of inorganic nutrient solution, five ml of algal suspension, a predetermined amount of organic material and enough distilled water to make the final volume 20 ml. Knops solution, adjusted to a pH of 7.2, was used as the nutrient medium (39). Each test contained control tubes and these were similar except they contained no organic chemicals. After the Knops solution, Appendix I, and distilled water were added, the tops of the test tubes were covered with the cap assembly and sterilized. Upon cooling, the required amount of organic compound was added under careful aseptic conditions. Following the addition of the test agent the algal suspension was added. The pH of the solution in the test tube was checked and reestablished at 7.0 using KOH. Finally this test tube assembly was attached to the gas manifold. Gas was released at a rate of about 60 to 120 bubbles per minute.

Each test was conducted over a period of five days. Daily analyses of algal suspensions were made.

The effect of the organic contaminant on the algae was determined by measuring changes in the chlorophyll content. The

chlorophyll pigment was extracted with methanol from a 2.5 ml sample. The algal suspension was centrifuged, washed and centrifuged again. The supernatant was discarded and the remaining packed cell material was coagulated in a hot water bath. From this mixture, the chlorophyll was extracted. Two extractions were required. The volume of the solution containing the chlorophyll was increased to 10 ml by adding an 80 per cent acetone-water mixture. The detailed procedure of the chlorophyll extraction method is given in Appendix II.

Special tests were conducted to make sure that there was no gross contamination. For this purpose, a nutrient media was prepared containing 500 ml of distilled water, 2.5 gm of glucose and 1 gm of tryptone or 0.5 gm of yeast extract. About 20 ml of the media was added to each test tube. After the test tubes were sterilized and cooled, about one ml of algal suspension was added from various experimental test tubes.

The inoculated test tubes containing the broth and algal suspension were incubated at 35±2°C for two days. If bacterial contamination was significant the broth would provide the nutrients for rapid growth. Such population increases might be observed by increases in turbidity. Minimum interference could be expected from the algal cells for they would settle to the bottom of the test tubes. This method proved to be fast and fairly reliable. Bimonthly tests were conducted to verify the laboratory procedures.

Most of the tests proved that the algal suspensions were not contaminated during the course of experimentation. Whenever the

tests indicated gross contamination the experiment was repeated for that particular concentration. Furthermore, the algal growth was almost unaffected when bacterial or other agents were present. Actually it did not seem to matter greatly if the test tube assemblies and nutrients were sterilized. These results are given in Fig. 3-2 and Appendix Tables III-1 and 2.

Algal Source

Bacteria-free cultures of Chlorella pyrenoidosa were obtained from Dr. J. E. Myers, Professor of Zoology and Botany and Director, Laboratory of Algal Physiology, The University of Texas. The growth was maintained so that the algal density remained at one gm/L. The chlorophyll content of the cells was about four mg/L.

The Standard Curve

A standard curve (Appendix Fig. IV-1) was plotted to establish the relationship between the optical density and chlorophyll content of algal cells. The detailed procedure is given in Appendix IV.

Organic Compounds

About sixty chemical compounds were investigated. Groups of organic chemicals were selected which were structurally similar, soluble in water and relatively non-volatile. The shorter chain fatty acids, dicarboxylic acids, alcohols, aldehydes and certain other miscellaneous compounds fulfilled the above requirements and hence were selected for this research.

Four or five different concentrations of the organic compounds were selected. The highest concentration was chosen so

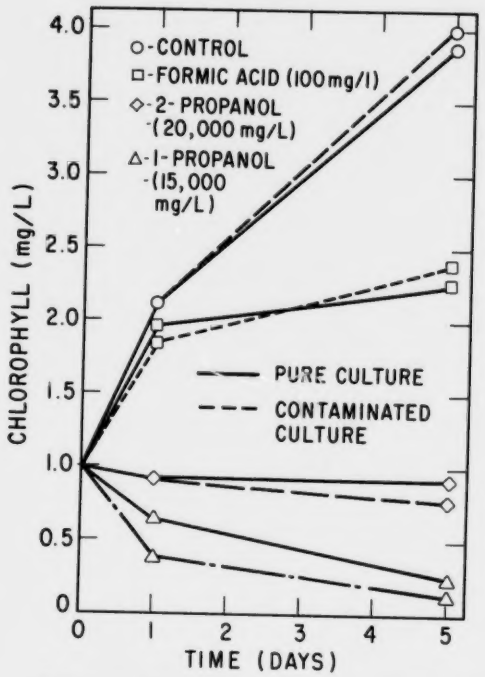

FIG. 3-2. EFFECTS OF 1 AND 2-PROPANOLS AND FORMIC ACID

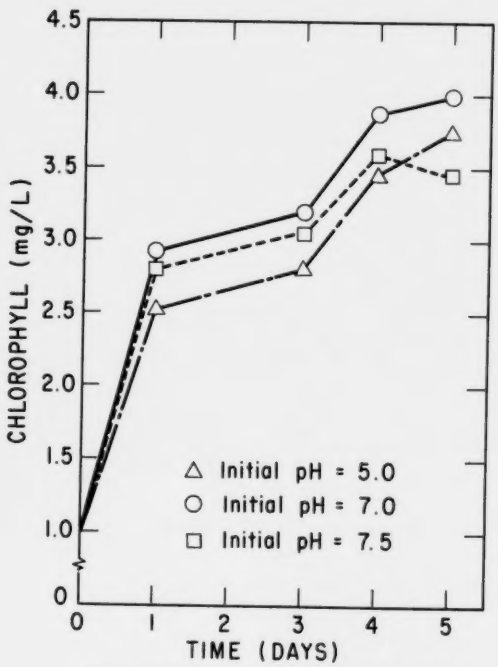

FIG. 3-3. ALGAL GROWTH AS A FUNCTION OF pH

that there would be 50 per cent reduction in the chlorophyll content of algal cells. Two or three more intermediate concentrations were selected to verify the 50 per cent value. Experience proved very important in selecting the best range of concentrations.

The physical constants of the organic chemicals tested are listed in Appendix Table V-1. The ingredients of the three commercial pesticides tested are shown in Appendix Table V-2.

Volatility Measurement Tests

One of the important criteria for the selection of organic compounds in this investigation was volatility. In spite of the careful screening of the chemicals, it was suspected that some of the alcohols and other compounds were lost in the test tubes through agitation and volatilization. The following experiment was designed to test and assess the percentage lost during experimentation.

To test volatility, duplicate test tubes containing 10 ml of each chemical were compared to 10 ml of distilled water. Otherwise, all test tubes were subjected to typical test conditions. The volume of the organic compounds and distilled water left in the various test tubes at the end of the five-day period was measured carefully and recorded as shown in Table 3-1.

The results of the experiment show that the loss of distilled water was from three to four per cent. Alcohols and fatty acids with one to three carbon atoms per molecule were relatively volatile. Aldehydes with three and four carbon atoms per molecule were also significantly volatile. The volume lost

Table 3-1. Results of Volatility Measurement Tests

Organic Compound (IUC Names)	Initial Volume (ml)	Final Volume (ml)	Loss (Per Cent)
Control (Distilled Water)	10	9.6	4.0
Fatty Acids			
Methanoic Acid	10	9.0	10.0
Ethanoic Acid	10	9.1	9.0
Propanoic Acid	10	9.2	8.0
Butanoic Acid	10	9.8	2.0
2-methyl propanoic Acid	10	9.8	2.0
Pentanoic Acid	10	9.85	1.5
3-methyl butanoic Acid	10	9.85	1.5
Hexanoic Acid	10	9.9	1.0
Heptanoic Acid	10	9.85	1.5
Octanoic Acid	10	9.85	1.5
Dicarboxylic Acids			
Ethanedioic Acid	10	9.8	2.0
Propanedioic Acid	10	9.8	2.0
Butanedioic Acid	10	9.85	1.5
Pentanedioic Acid	10	9.85	1.5
Hexanedioic Acid	10	9.85	1.5
Heptanedioic Acid	10	9.85	1.5
Control (Distilled Water)	10	9.7	3.0
Alcohols			
Methanol	10	6.5	35.0
Ethanol	10	7.7	23.0
1-propanol	10	7.85	21.5
2-propanol	10	7.8	22.0
1-butanol	10	9.85	1.5
2-butanol	10	9.85	1.5
3-butanol	10	9.8	2.0
1-hexanol	10	9.9	1.0
2-hexanol	10	9.9	1.0
Heptanol	10	9.9	1.0
Octanol	10	9.9	1.0
Miscellaneous Acids			
Propenoic Acid	10	9.8	2.0
Butenoic Acid	10	9.8	2.0
Hydroxyethanoic Acid	10	9.85	1.5
2,3-dihydroxybutanedioic Acid	10	9.8	2.0
Sulfanilic Acid	10	9.85	1.5
Methoxyethanoic Acid	10	9.75	2.5
2-oxopropanoic Acid	10	9.8	2.0
Ethanoic Anhydride	10	9.8	2.0

Table 3-1 (continued)

Organic Compound (IUC Names)	Initial Volume (ml)	Final Volume (ml)	Loss (Per Cent)
Aldehydes			
1-propanal	10	8.0	20.0
1-butanal	10	8.85	11.5
2-methyl propanal	10	8.85	11.5
1-heptanal	10	9.7	3.0
Glycols			
1,2-ethanediol	10	9.8	2.0
1,2-propanediol	10	9.8	2.0
Phenols			
Phenol	10	9.8	2.0
Cresol	10	9.8	2.0
Commercial pesticides			
Malthane (Trade Name)	10	9.7	3.0
Ortho (Trade Name)	10	9.7	3.0
DDT in Xylene	10	9.7	3.0

during five days varied from eight to 35 per cent for these particular alcohols, fatty acids, and aldehydes. Hence corresponding correction factors were introduced while calculating the toxic concentrations of the above-mentioned chemicals. Most of the other chemicals were relatively non-volatile. The correction factors were not used when the loss of chemical was less than in controls, namely, 3.5 per cent, Table 3-1.

Effect of pH on Algal Growth

Some environmental factors could be controlled, but pH changes necessarily occurred. In some cases, the final pH dropped to as low as 5.0. This pH drop was especially prevalent in the test tubes in which the algae were destroyed. In order to measure the effect of pH on algal growth, the following experiments were conducted.

The changes in chlorophyll content of the algal cells were measured as a function of pH. The Knops solution was prepared and the pH was 3.0 before adding KOH. The Knops solution was divided into five separate parts and KOH was added until the pH of the test solution increased to 5.0, 5.5, 6.0, 7.0 and 7.5. Thirty test tubes were used in this experiment each containing five ml algae and 10 ml distilled water. All tubes contained five ml of Knops solutions of different pH.

The results of the experiment are shown in Fig. 3-3 and Table 3-2. The chlorophyll concentration in the control test tube (pH=7.0) was 4.0 mg/L after five days. The range of chlorophyll concentrations in the other test tubes varied from 3.5 to 4.3 mg/L after five days. In none of the test tubes was the

chlorophyll destroyed to any significant extent. Thus, a pH value as low as 5.0 was not in itself responsible for the reduction in chlorophyll concentration in the various toxicity tests. The algal systems were able to adjust the pH within the range between 6.4 and 7.0.

Table 3-2. Algal Growth as a Function of pH.

Initial pH	Transmittance (Per cent)				Final pH
	After 1 Day	After 3 Days	After 4 Days	After 5 Days	
5.0	81	79	74	72	6.5
5.5	80	78	75	74	6.5
6.0	79	75	70	68	6.6
7.0	78	76	71	70	6.4
7.5	79	77	73	74	7.0

Inactivation Study

As discussed in the literature review, the anti-metabolite effects of some toxic chemicals were found to be inactivated by the corresponding metabolic intermediates of photosynthesis. In order to study the inactivation capacities of these metabolic products towards toxic materials, experiments were conducted as explained below.

After approximate toxicity ranges had been established, special inactivation studies were undertaken. About 45 culture tubes were used. Five tubes were used as controls containing five ml of algal solution, five ml of Knops solution and 10 ml of distilled water. In other test tubes the same amounts of algal suspension and Knops solution were added. About 15 tubes contained

the toxic organic chemicals. To the rest of the culture tubes the toxic chemicals as well as the corresponding metabolic intermediates were added. Each test was conducted for the usual five days, and the changes in chlorophyll content of the algal cells were measured.

Table 3-3 lists the toxic chemicals and the corresponding inactivators. These inactivators were selected based on the studies conducted by other invetigators as explained below.

Lefrancois and Quellet (28) showed that ethanol interfered in the photosynthetic metabolism of Scenedesmus by suppressing the formation of PGA. Hence, a test was conducted to investigate if PGA could neutralize the toxic effects of ethanol to Chlorella pyrenoidosa. Another test was performed with methanol and 1-propanol as the toxic chemicals and PGA as the toxicity inactivator to verify if the above-mentioned theory could be extended to the other alcohols.

Table 3-3. List of Toxic Chemicals and Corresponding Inactivators Used

Toxic Chemical	Toxicity Inactivator
Methanol	Phospho Glyceric Acid
Ethanol	Phospho Glyceric Acid
1-Propanol	Phospho Glyceric Acid
o-Phenanthroline	Phospho Glyceric Acid
o-Phenanthroline	Ferrous Sulfate
2-Thiouracil	Uracil
2-Thiouracil	Adenine
2-Thiouracil	Hypoxanthine
2-Thiouracil	Orotic Acid
2-Thiouracil	Cytidylic Acid
CCCP	Fructose-1,6-Diphosphate

The toxicity of o-phenanthroline which interferes in the photosynthetic metabolism is explained by two different theories. Lefrancois and Quellet (28) proposed that o-phenanthroline acted in a similar manner to ethanol. However, Fry and Pietrs (14) showed that the addition of o-phenanthroline to pyridine nucleotide reductase resulted in the loss of iron and sulfur from the enzyme. Therefore two experiments were conducted each with o-phenanthroline as the toxic chemical. In the first test, PGA was added as the toxicity inactivator. In the second test, the concentration of ferrous sulfate was increased in the Knops solution to verify if the excess available ferrous sulfate could neutralize the toxic effects of o-phenanthroline.

2-thiouracil is the competitive inhibitor of photosynthetic metabolism (51). The pyrimidine derivatives, uracil, orotic acid and cytidylic acid, and the purine derivatives, adenine and hypoxanthine, were used by Shimeno and Shukuo (46) as possible inactivators of toxicity. Hence, similar inactivation studies were conducted with Chlorella pyrenoidosa as the test organism.

According to Bamberger, et al. (5), the toxic effects exhibited by the chemical CCCP on spinach chloroplasts were minimized in the presence of fructose-1,6 diphosphate. Hence, a similar test was conducted to verify if the inactivation theory could be applicable in the case of algal metabolism.

CHAPTER IV

EVALUATION OF RESULTS

The evaluation and interpretation of results consisted of defining the toxic ranges of the chemicals and comparing the toxic concentrations of the chemicals. Various groups of short chain fatty acids, dicarboxylic acids, alcohols, aldehydes and miscellaneous compounds were studied. The toxic characteristics of the compounds were related with the molecular structure.

The results of the inactivation tests were evaluated in comparison with the results of similar experiments conducted by other investigators.

The entire test data are shown in Tables 4-1 to 4-20. The chlorophyll production as a function of time at different concentrations of the toxic chemicals are shown in Figs. 4-1 to 4-30. For clarity, only certain selected typical and representative curves are shown in these figures.

Defining the Toxic Concentration

The toxic concentration was arbitrarily defined as that concentration of organic material which caused a reduction of at least 50 per cent in the chlorophyll content of the algal cells after five days of contact. The initial concentration of chlorophyll in the test solution was 4 mg/L.

Short Chain Fatty Acids

The fatty acids include a large series of straight chain acids that begin with methanoic acid and continue to compounds with more than 20 carbon atoms (33). The ten short chain fatty

acids tested were toxic to Chlorella pyrenoidosa at different minimum concentrations. The results of the test conducted on these fatty acids are given in Table 4-1 and Figs. 4-1 through 4-6. Heptanoic acid, which was the most effective of all the fatty acids in reducing the chlorophyll content of algal cells, was toxic to algae at concentrations above 200 mg/L. 3-methyl butanoic acid, which was the least effective in reducing the chlorophyll content, was toxic at concentrations beginning at 400 mg/L.

The first three members of the fatty acids, namely, methanoic, ethanoic and propanoic acids, were significantly volatile, Table 3-1. Hence a correction factor was introduced in determining the actual toxic concentrations of these three fatty acids. The remaining seven fatty acids which had more than three carbon atoms per molecule were less volatile than the distilled water in the control. Hence, correction factors were not introduced for these seven fatty acids.

A general relationship did exist between the toxic characteristics and the number of carbon atoms per molecule of the fatty acids. The straight chain fatty acids with odd number of carbon atoms appeared to be more toxic than the corresponding acids with even number of carbon atoms, Fig. 4-6.

The straight chain fatty acids with four and five carbon atoms (butanoic and pentanoic acids) were more toxic than the corresponding branched chain acids, Figs. 4-2, 4-3 and 4-5. The volatile characteristics of these four acids were not responsible for the above-mentioned relationship. The loss of butanoic acid and 2-methyl propanoic acid were equally low (two

Table 4-1. Test Data for Fatty Acids

Chemical Tested	Concentration of Chemical (mg/L) Without Volatility Correction Factors	Concentration of Chemical (mg/L) With Volatility Correction Factors	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Methanoic	Control	-	78	71	70	72	72	6.7
	50	45	78	75	73	70	74	6.6
Acid	100	90	80	71	73	78	79	6.6
	200	180	85	72	88	86	87	6.5
	250	225	98	95	99	98	98	6.2
	300	270	100	100	100	100	100	5.9
Ethanoic	Control	-	77	71	70	70	72	6.8
	100	90	79	68	71	65	70	6.5
Acid	200	180	80	70	72	71	70	6.3
	300	270	83	79	85	75	81	6.3
	350	320	88	88	90	91	90	6.0
	400	360	99	99	99	99	100	5.8
Propanoic	Control	-	77	72	72	70	70	6.6
	100	90	85	84	79	79	83	6.6
Acid	200	180	93	90	91	89	89	6.7
	250	230	96	94	92	91	93	6.2
	300	275	99	100	100	100	100	6.3
	400	370	100	100	100	100	100	6.0
Butanoic	Control	-	77	71	70	71	70	6.7
	200	200	88	80	80	76	71	6.6
Acid	250	250	92	92	83	73	72	6.6
	300	300	93	97	95	82	83	6.4
	325	325	94	98	96	90	92	6.3
	350	350	95	100	100	100	100	6.0

Table 4-1 (continued)

Chemical Tested	Concentration of Chemical (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
2-methyl propanoic Acid	Control	78	70	71	71	72	6.5
	200	71	70	71	72	70	6.5
	250	87	70	71	71	70	6.5
	300	85	74	-	73	72	6.6
	325	90	91	91	85	85	6.2
	350	100	100	100	100	100	6.1
Pentanoic Acid	Control	79	72	70	72	71	6.7
	100	88	92	89	78	79	6.7
	200	89	94	92	80	86	6.6
	250	91	95	93	92	90	6.0
	300	95	97	96	99	100	6.1
	400	97	99	100	100	100	5.1
3-methyl butanoic Acid	Control	79	72	70	72	71	6.6
	100	85	80	70	71	71	6.4
	200	87	79	72	-	83	6.5
	300	91	85	76	-	83	6.4
	400	94	92	94	95	96	5.7
Hexanoic Acid	Control	77	72	70	72	72	6.5
	200	87	90	86	77	79	6.4
	300	92	91	95	93	94	6.0
	350	95	96	98	99	100	5.2
	400	98	100	100	100	100	5.1
Heptanoic Acid	Control	78	72	70	70	70	6.9
	50	82	81	85	74	75	6.8
	100	88	95	89	72	84	6.8
	150	91	98	90	82	90	6.7
	200	93	100	100	100	100	6.1

Table 4-1 (continued).

Chemical Tested	Concentration of Chemical (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Octanoic Acid	Control	78	72	71	70	70	6.9
	50	88	87	80	71	79	6.5
	100	90	90	85	70	78	6.4
	150	93	97	90	80	80	6.3
	200	90	91	92	93	94	6.0
	250	93	99	100	100	100	5.9

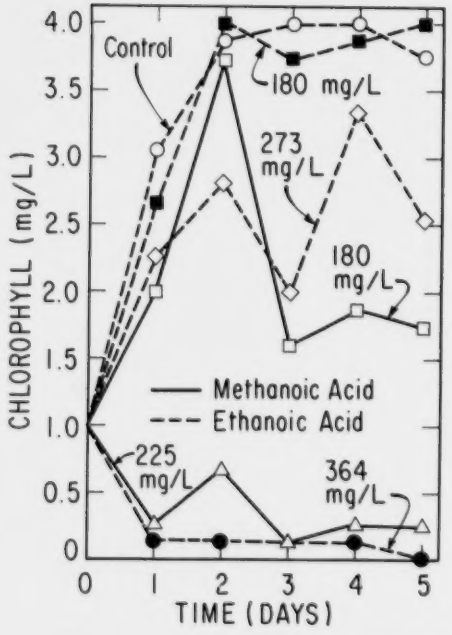

FIG. 4-1. EFFECTS OF METHANOIC AND ETHANOIC ACIDS

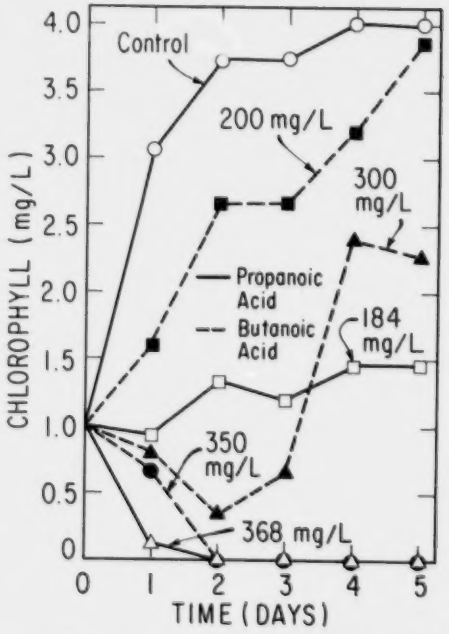

FIG. 4-2. EFFECTS OF PROPANOIC AND BUTANOIC ACIDS

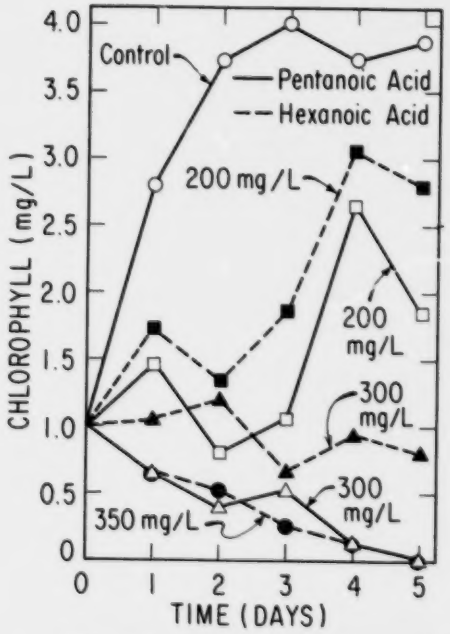

FIG. 4-3. EFFECTS OF PENTANOIC AND HEXANOIC ACIDS

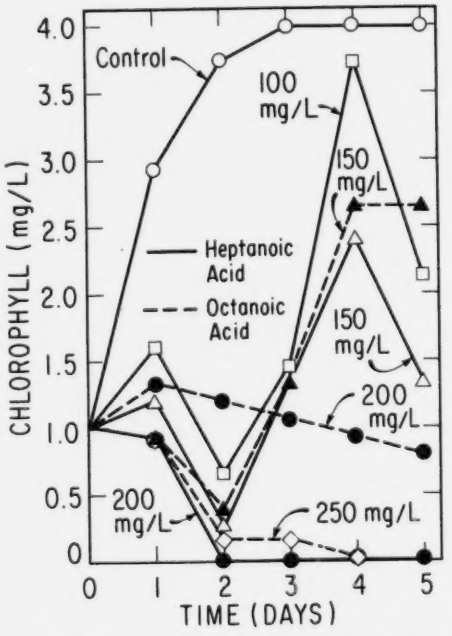

FIG. 4-4. EFFECTS OF HEPTANOIC AND OCTANOIC ACIDS

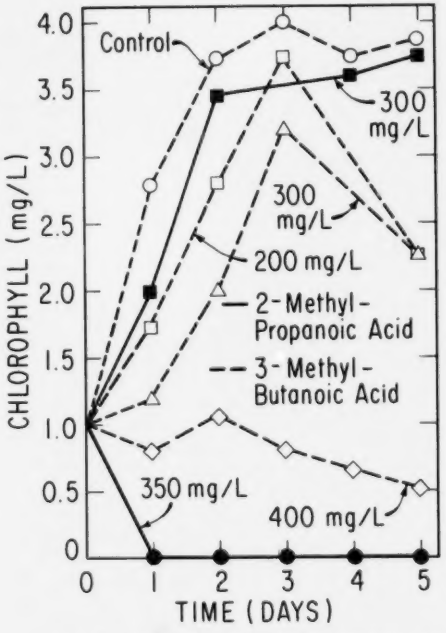

FIG. 4-5. EFFECTS OF BRANCHED CHAIN FATTY ACIDS

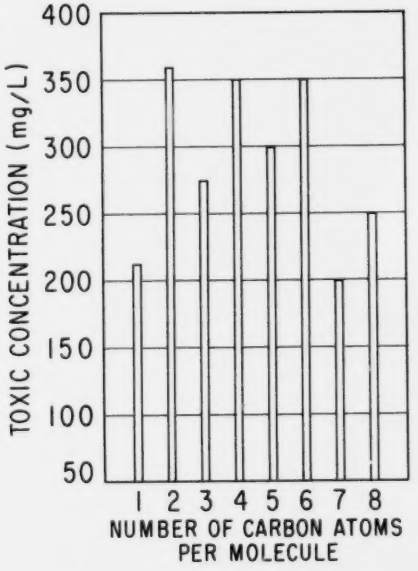

FIG. 4-6. TOXICITY OF FATTY ACIDS

per cent) as shown in the volatility measurement test. Similarly, the loss of pentanoic acid and 3-methyl butanoic acid were equally low (1.5 per cent).

At a concentration of 300 mg/L of butanoic acid, the chlorophyll content was 2.1 mg/L after five days of contact. Whereas, at the same concentration of 300 mg/L of 2-methyl propanoic acid, the chlorophyll content was about 3.7 mg/L after five days of exposure.

Pentanoic acid was toxic at concentrations above 300 mg/L. However, 3-methyl butanoic acid, the corresponding branched chain fatty acid, was toxic to algae only above 400 mg/L. At a given concentration of 300 mg/L, pentanoic and 3-methyl butanoic acids changed the chlorophyll content respectively to zero mg/L and 2.2 mg/L at the end of the test period. Similar differences could be noticed at concentrations of 100 mg/L and 200 mg/L. Thus a difference did exist between the toxic behaviors of straight chain fatty acids and the corresponding branched chain acids.

Dicarboxylic Acids

The six dicarboxylic acids were toxic to algae at different concentrations, Table 4-2 and Figs. 4-7 through 4-9. Ethanedioic acid and butanedioic acid were respectively the most toxic and the least toxic compounds in the group. Toxic conditions became evident at 300 mg/L for ethanedioic acid. However, butanedioic acid was not toxic until a concentration of 2,500 mg/L was reached.

The toxic effects of these acids decreased with increasing number of carbon atoms per molecule for the first three acids in the chain, Fig. 4-10. However, the toxic effects increased with

Table 4-2. Test Data for Dicarboxylic Acids

Chemical Tested	Concentration of Chemical (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Ethanedioic Acid	Control	80	73	70	71	70	6.8
	100	79	76	74	72	73	6.7
	200	93	93	78	78	80	6.7
	300	93	94	97	99	98	6.2
	400	95	-	99	-	100	6.3
Propanedioic Acid	Control	80	73	70	71	70	6.8
	300	85	-	84	82	73	6.5
	400	90	-	89	93	95	6.3
	500	91	93	95	96	97	6.1
	600	94	94	94	99	100	6.1
Butanedioic Acid	Control	77	70	72	71	70	6.7
	1,000	80	81	82	83	85	6.6
	1,500	82	-	89	90	91	6.6
	2,000	88	-	93	99	94	6.0
	2,500	90	93	96	99	100	5.9
Pentanedioic Acid	Control	77	70	72	71	70	6.6
	500	80	75	74	75	76	6.5
	1,000	79	81	86	91	93	6.4
	1,500	89	96	100	100	100	6.2
	2,000	-	100	100	100	100	5.9
Hexanedioic Acid	Control	80	72	71	70	71	6.6
	500	-	83	82	68	70	6.0
	750	86	89	90	92	94	5.3
	1,000	88	94	94	96	97	5.2
	1,250	90	99	100	98	99	5.0

Table 4-2 (continued)

Chemical Tested	Concentration of Chemical (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Heptanedioic Acid	Control	80	72	71	70	71	6.6
	250	-	68	67	67	68	6.4
	500	85	79	85	80	78	6.2
	750	95	96	97	98	98	5.2
	1,000	100	98	100	99	99	5.2

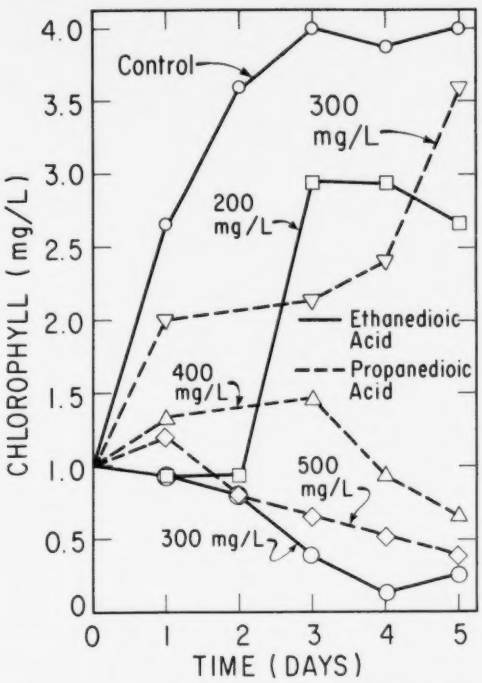

FIG. 4-7. EFFECTS OF ETHANEDIOIC AND PROPANEDIOIC ACIDS

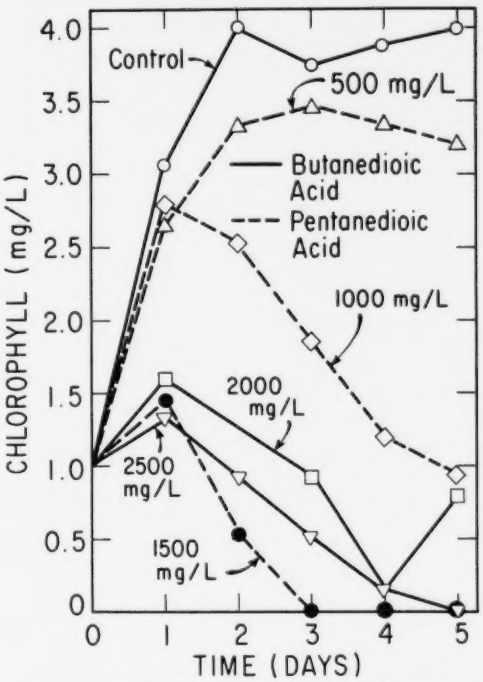

FIG. 4-8. EFFECTS OF BUTANEDIOIC AND PENTANEDIOIC ACIDS

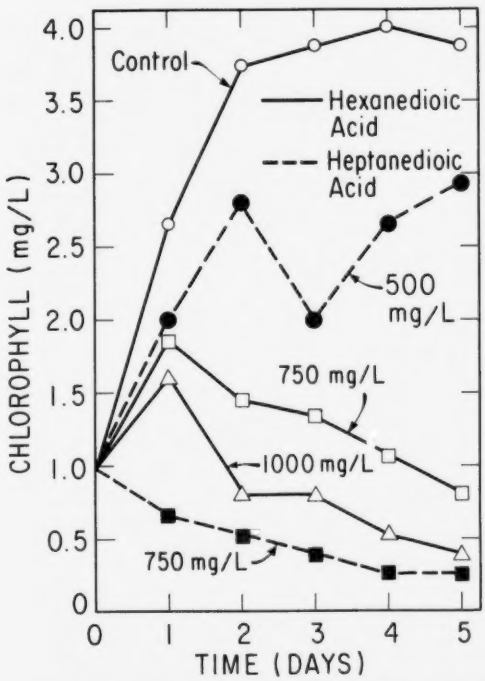

FIG. 4-9. EFFECTS OF HEXANEDIOIC AND HEPTANEDIOIC ACIDS

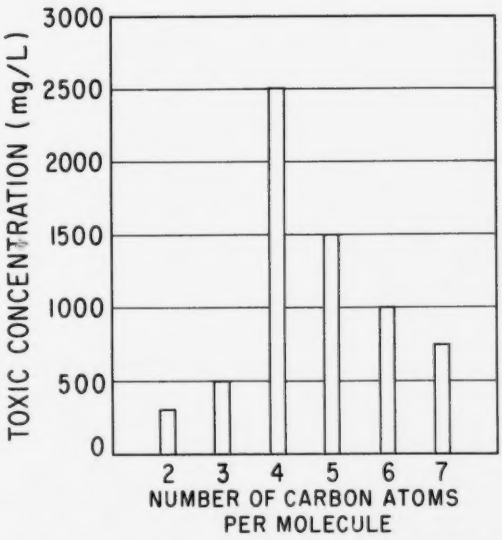

FIG. 4-10. TOXICITY OF DICARBOXYLIC ACIDS

increasing number of carbon atoms for the fourth, fifth, and sixth members, Fig. 4-10.

None of the dicarboxylic acids were influenced significantly by volatility. The loss of these acids by volatilization was between 1.5 and 2.0 per cent which was less than that of the distilled water in the control tubes, Table 3-1. Hence, correction factors were not necessary for the dicarboxylic acids.

In the presence of 300 mg/L of ethanedioic acid, the chlorophyll content decreased to 0.24 mg/L at the end of the test period. In the presence of the same concentration of propanedioic acid, the chlorophyll content increased to 3.58 mg/L at the end of the test period. The results indicated that the two-carbon dicarboxylic acid, ethanedioic acid, was more toxic to algae than the three-carbon acid, propanedioic acid.

The results of the experiment with pentanedioic acid showed that the toxic chemical completely destroyed the photosynthetic pigment at the concentration of 1,500 mg/L after three days of contact. However, in the presence of 2,000 mg/L of pentanedioic acid, the chlorophyll content was completely destroyed after two days of contact. Thus the minimum toxic concentration of a chemical was time-dependent. Similar examples could be noted for most of the organic compounds.

The presence of 750 and 1,000 mg/L of hexanedioic acid did not reduce the concentration of chlorophyll for the first 24 hour period. However, during the continued contact of the chemical, the photosynthetic pigment was gradually reduced and there was no recovery from this reduction.

Heptanedioic acid was toxic to the test algae at concentrations beginning at 750 mg/L. The presence of 750 mg/L of heptanedioic acid influenced the chlorophyll reduction on the first day itself and continued the destruction throughout the test period until the concentration of the green pigment was reduced to 0.26 mg/L.

Alcohols

Seven primary alcohols, three secondary alcohols and one tertiary alcohol are included in this group. These alcohols were toxic to algae at different concentrations, Table 4-3 and Figs. 4-11 through 4-15. Toxic conditions were evident at 250 mg/L for octanol. However, methanol was not toxic until a concentration of 32,500 mg/L was reached. The remaining alcohols in the group were more toxic than methanol and less toxic than octanol.

The toxicity of alcohols to the test alga increased with increasing number of carbon atoms in the molecule, Fig. 4-16. These results are in agreement with the findings of Lefrancois and Quellet (22) who used Scenedesmus as the test organism. They tested four alcohols. The present study indicates that the conclusions of Lefrancois and Quellet can be extended to include three more alcohols in the chain.

The number of carbon atoms in the alcohol molecules, either odd or even, exhibited no significantly different toxic characteristics. Whereas in fatty acids the toxicity was dependent on whether the number of carbon atoms were odd or even.

The two primary alcohols, 1-propanol and 1-hexanol, were more toxic than the corresponding secondary alcohols, 2-propanol and

Table 4-3. Test Data for Alcohols

Chemical Tested	Concentration (mg/L) Without Correction Factors	Concentration (mg/L) With Correction Factors	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Methanol	Control	-	80	75	71	-	70	6.8
	40,000	26,000	85	85	86	-	86	6.9
	50,000	32,500	86	90	97	98	99	6.7
	60,000	38,000	91	95	97	98	99	6.6
	70,000	45,500	91	98	100	100	100	6.3
Ethanol	Control	-	80	-	75	70	70	7.0
	15,000	11,550	88	-	85	84	72	6.9
	30,000	23,100	95	-	96	97	87	7.0
	37,500	28,875	99	99	99	100	100	6.3
	45,000	34,650	100	100	100	100	100	6.3
1-Propanol	Control	-	83	-	72	70	70	6.7
	10,000	7,850	94	89	84	85	86	6.5
	15,000	11,775	95	96	97	97	98	6.4
	20,000	15,700	100	100	100	100	100	6.2
2-Propanol	Control	-	83	-	72	70	70	6.7
	10,000	7,800	90	-	79	80	81	6.5
	15,000	11,700	92	88	84	86	88	6.5
	20,000	15,600	93	93	93	94	93	6.4
	25,000	19,500	94	-	95	97	100	6.1

Table 4-3 (continued)

Chemical Tested	Concentration of Chemical (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
1-Butanol	Control	78	70	70	70	70	6.7
	4,000	86	85	88	87	84	6.5
	6,000	92	93	97	90	91	6.3
	8,000	94	98	96	96	95	5.9
	10,000	97	100	100	100	100	5.2
2-Butanol	4,000	78	72	72	71	70	6.5
	6,000	91	91	92	84	83	6.3
	8,000	95	99	99	99	93	5.8
	10,000	98	100	100	100	100	5.3
3-Butanol	Control	75	-	77	73	70	6.8
	10,000	87	83	80	78	77	6.8
	20,000	94	91	88	85	82	6.8
	25,000	96	95	94	97	99	6.7
	30,000	97	-	100	100	100	6.6
1-Hexanol	Control	77	72	72	71	70	6.5
	750	70	71	80	79	78	6.6
	900	88	89	84	-	83	6.5
	1,050	92	93	90	90	90	5.9
	1,200	92	-	91	-	92	5.2
	1,350	99	100	100	100	100	5.0
2-Hexanol	Control	80	70	-	71	70	6.5
	1,600	85	78	75	72	70	6.6
	2,400	93	84	-	75	76	6.5
	3,200	94	88	89	90	98	5.1
	4,000	100	100	100	100	100	5.0

Table 4-3 (continued)

Chemical Tested	Concentration of Chemical (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Heptanol	Control	78	73	72	72	71	6.5
	375	74	72	80	-	81	6.5
	450	75	74	85	-	86	6.4
	525	93	92	95	-	96	5.3
	600	98	100	100	100	100	5.1
Octanol	Control	79	76	73	72	70	6.6
	100	78	77	75	73	71	6.6
	150	80	82	84	84	85	6.5
	200	84	87	90	92	93	6.1
	250	85	90	94	95	96	5.7

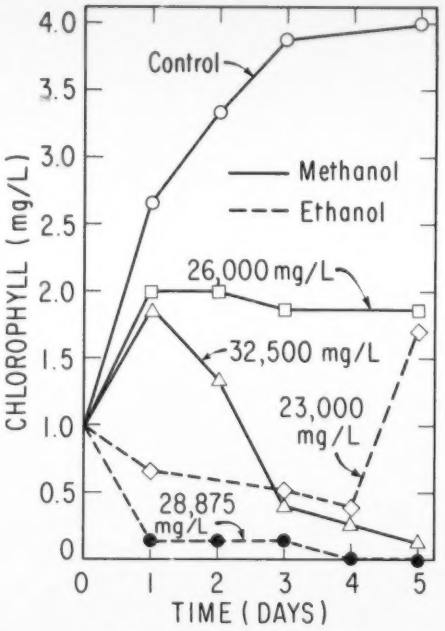

FIG. 4-11. EFFECTS OF METHANOL AND ETHANOL

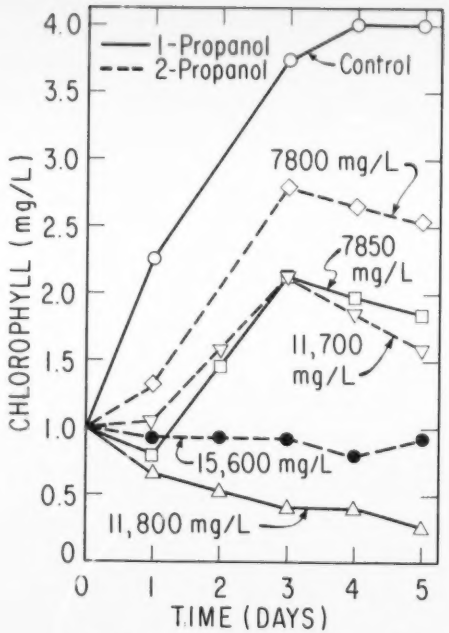

FIG. 4-12. EFFECTS OF 1 AND 2-PROPANOLS

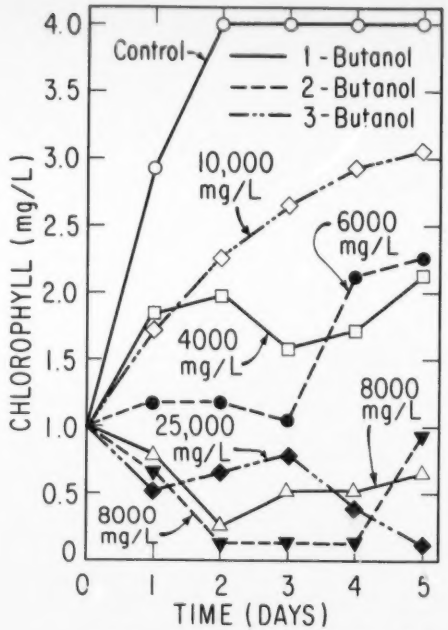

FIG. 4-13. EFFECTS OF 1, 2 AND 3-BUTANOLS

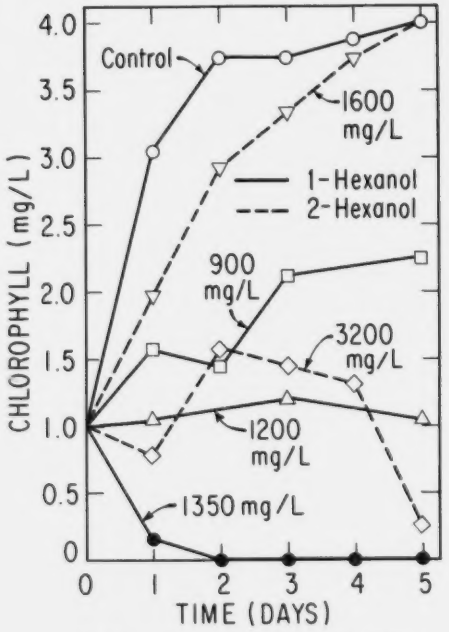

FIG. 4-14. EFFECTS OF 1 AND 2-HEXANOLS

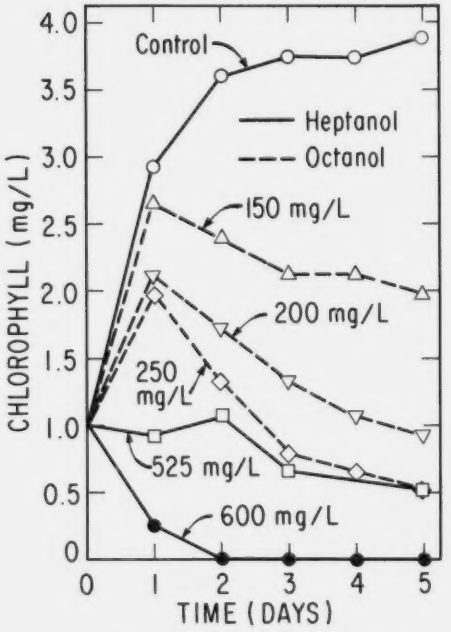

FIG. 4-15. EFFECTS OF HEPTANOL AND OCTANOL

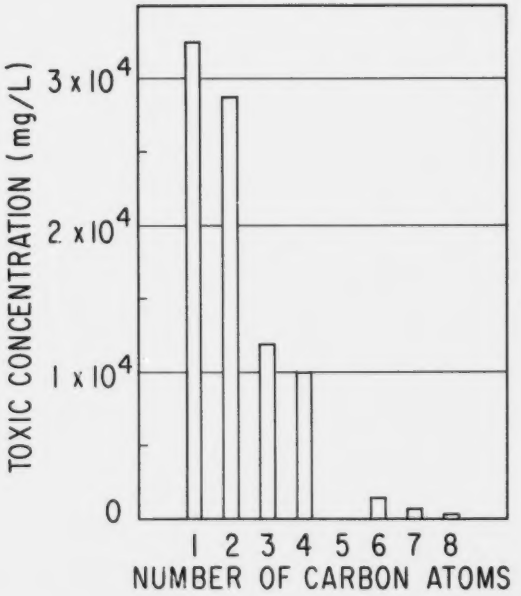

FIG. 4-16. TOXIC CONCENTRATIONS OF PRIMARY ALCOHOLS

2-hexanol, Fig. 4-12 and 4-14. Similarity of the above characteristic can be noted with the fatty acids. Volatile characteristics of these four alcohols did not influence the above-mentioned relationship. The results of the volatility measurement test showed that 1-propanol and 2-propanol were lost by volatilization at the rate of 21.5 and 22 per cent respectively in five days. 1-propanol was toxic to the test algae at concentrations at and above 11,800 mg/L. However, the toxicity of 2-propanol was not apparent until a concentration of 19,500 mg/L was reached.

Both 1-hexanol and 2-hexanol were less volatile than the distilled water in the control. Hence a correction factor to include the volatile characteristic was not needed. 1-hexanol completely destroyed the chlorophyll content of the algal cells at concentrations as low as 1,350 mg/L. However 2-hexanol at a much higher concentration of 3,200 mg/L only reduced the chlorophyll concentration by about 80 per cent.

As an exception to the above relationship, 1-butanol was not more toxic to algae than 2-butanol, Fig. 4-13. However, 1-butanol and 2-butanol were more toxic than 3-butanol, Fig. 4-13. At a concentration of 10,000 mg/L, 1-butanol and 2-butanol completely eliminated the chlorophyll in the algal cells by the second day and there was no recovery. Yet, at the same concentration of 10,000 mg/L of 3-butanol, the chlorophyll content was 2.9 mg/L after five days. These three alcohols with four carbon atoms per molecule were less volatile than the distilled water in control, Table 3-1.

Miscellaneous Organic Compounds

The organic compounds discussed in this section consist of eight organic acids, four aldehydes, two glycols, two phenols, and three commercial pesticides.

The changes in chlorophyll content of algal cells in the presence of various concentrations of the organic acids are shown in the Figs. 4-17 through 4-24 and Tables 4-4 through 4-9. These organic acids were toxic to the test alga at different concentrations beginning between 150 and 3,000 mg/L.

The unsaturated organic acids, propenoic and butenoic acids, were the most toxic of these eight organic acids. Propenoic acid and butenoic acid were toxic to algae at the concentrations beginning at 150 and 300 mg/L, respectively, Fig. 4-17.

The two hydroxy acids, 2,3-dihydroxy butanedioic and hydroxyethanoic, were toxic to algae at concentrations beginning at 500 and 3,000 mg/L, respectively.

At a concentration of 900 mg/L of sulfanilic acid, the chlorophyll content was reduced to 0.24 mg/L after three days of contact, Fig. 4-18. However, a significant recovery in the formation of the green pigment was noticed at the end of the test period. Similar recoveries were observed for hydroxyethanoic acid and 2-oxopropanoic acid (pyruvic acid) at a concentration of 500 mg/L, Figs. 4-17 and 4-19. The resistance developed by the test organism may be the cause for such recoveries.

Aldehydes with three and four carbons in the chain were more volatile than distilled water, Table 3-1. Hence, corresponding correction factors were introduced to take into account the vola-

Table 4-4. Test Data for Miscellaneous Organic Acids

Chemical Tested	Concentration (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Propenoic Acid (Acrylic Acid)	Control	80	72	70	72	72	6.8
	25	87	96	96	93	87	6.7
	50	90	94	97	95	93	6.6
	100	93	90	96	96	94	6.7
	150	96	95	100	100	100	6.0
	200	97	97	99	100	100	5.9
Butenoic Acid (Crotonic Acid)	Control	70	70	70	70	72	6.7
	100	75	75	75	75	74	6.6
	200	76	-	72	72	76	6.6
	250	80	81	82	84	89	6.2
	300	100	100	100	100	100	5.8
Hydroxyethanoic Acid	Control	78	72	71	71	70	6.7
	2,000	93	92	91	90	84	6.7
	2,500	99	98	99	98	95	6.6
	3,000	100	100	100	100	98	6.4
	3,500	100	100	100	100	100	6.1
2,3-dihydroxybutanedioic Acid (Tartaric Acid)	Control	79	72	72	72	72	6.6
	200	81	79	78	-	78	6.6
	300	84	77	-	74	78	6.5
	400	90	90	87	86	87	6.6
	500	94	98	98	97	98	6.0
	1,000	94	-	99	98	100	5.8
Sulfanilic Acid	Control	80	71	70	70	70	6.6
	750	88	86	78	75	72	6.5
	900	90	97	98	86	83	6.0
	1,050	93	9	99	99	100	5.7

Table 4-4 (continued)

Chemical Tested	Concentration (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Methoxy ethanoic Acid	Control	80	72	72	70	73	6.7
	300	86	-	70	70	73	6.6
	400	-	83	72	70	74	6.5
	500	91	90	91	90	89	6.2
	600	92	93	95	97	98	6.0
2-oxo propanoic Acid	Control	77	72	70	70	70	6.7
	250	79	75	70	79	70	6.6
	500	94	93	94	94	84	6.6
	1,000	96	98	100	100	100	6.2
	1,500	100	99	100	100	100	6.1
Ethanoic Anhydride	Control	78	72	70	70	70	6.6
	50	88	77	-	76	75	6.5
	100	89	77	-	74	79	6.6
	250	93	86	83	77	79	6.5
	300	94	89	87	85	92	6.0
	400	98	97	97	98	99	5.3

Table 4-5. Test Data for Aldehydes

Chemical Tested	Concentration (mg/L) Without Correction Factors	Concentration (mg/L) With Correction Factors	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Propanal	Control	-	75	71	71	71	70	6.8
	2,000	1,600	82	81	80	75	73	6.6
	3,000	2,400	-	91	-	85	82	6.6
	3,500	2,800	-	90	95	94	90	6.2
	4,000	3,200	89	95	96	96	92	6.1
	4,500	3,600	96	96	96	97	99	5.8
1-butanal	Control	-	78	72	71	70	72	6.8
	1,500	1,328	87	85	87	84	80	6.5
	2,000	1,770	85	86	87	87	88	6.5
	2,500	2,213	90	88	90	91	89	6.6
	3,000	2,655	95	94	100	100	100	6.2
2-methyl propanal	Control	-	77	71	72	71	72	6.8
	2,000	1,770	83	81	84	82	84	6.5
	2,500	2,213	85	81	85	81	84	6.5
	3,000	2,655	93	90	91	92	90	6.4
	4,000	3,540	94	94	92	93	97	6.1
1-heptanal	Control	-	79	72	72	71	70	6.5
	90	-	80	76	-	72	74	6.5
	120	-	82	80	-	75	79	6.4
	150	-	85	82	81	79	80	6.5
	180	-	86	85	-	85	88	6.2
	250	-	92	94	95	96	98	5.7

Table 4-6. Test Data for Glycols

Chemical Tested	Concentration (mg/L)	Transmittance (Per Cent) After 1 Day	After 2 Days	After 3 Days	After 5 Days	Final pH
1,2-ethane-diol	Control	79	74	72	71	6.5
	100,000	85	87	89	88	6.5
	150,000	86	88	92	90	6.4
	200,000	88	90	96	99	6.3
	300,000	92	98	100	100	6.0
	400,000	96	99	100	100	5.9
1,2-propanediol	Control	78	74	72	71	6.5
	50,000	80	85	84	92	6.4
	100,000	92	94	98	99	6.4
	200,000	95	97	99	100	6.0
	300,000	100	99	100	100	6.1

Table 4-7. Test Data for Phenol

Concentration (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Control	80	70	72	70	71	6.5
750	-	91	90	85	85	6.4
1,000	89	91	93	90	95	6.3
1,250	90	92	94	98	100	6.0
1,500	90	95	98	100	100	5.8

Table 4-8. Test Data for Cresol

Concentration (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Control	79	77	76	75	74	6.7
210	80	78	78	79	80	6.4
420	83	80	82	-	85	6.3
840	90	90	90	93	97	6.3
1,680	94	95	97	99	100	5.9

Table 4-9. Test Data for Commercial Pesticides

Chemical Tested	Concentration (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Malthane (6% DDD 6% Malathion 84% Xylene)	Control	70	71	67	65	67	6.3
	70	74	75	82	86	88	6.4
	140	87	89	98	-	95	6.5
	210	95	99	100	100	100	6.3
	280	100	100	100	100	100	6.6
Ortho (29% Malathion, 10% BHC 43% Solvent 18% Inert Ingredients)	Control	71	77	63	67	66	6.8
	34	72	76	63	-	63	6.7
	68	79	79	64	-	69	6.5
	134	-	82	65	75	71	6.5
	202	87	85	79	78	78	6.7
	340	95	100	99	100	99	6.8
25% DDT 67% Xylene 8% Emulsifier	Control	70	71	66	67	67	6.6
	50	71	75	74	67	62	6.4
	62.5	75	76	82	82	82	6.2
	100	78	81	82	83	87	6.1
	125	99	99	100	100	100	5.9

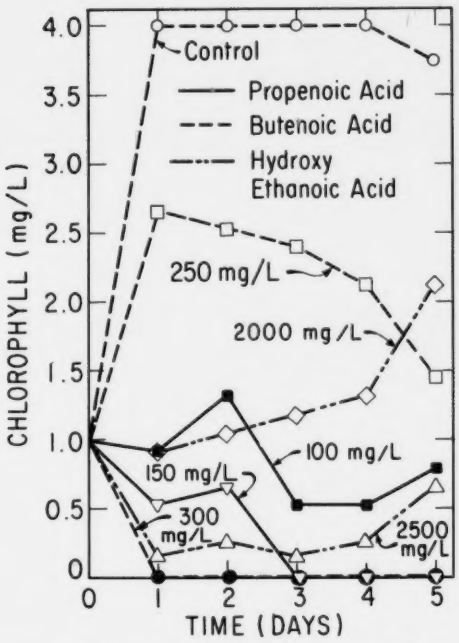

FIG. 4-17. EFFECTS OF PROPENOIC, BUTENOIC AND HYDROXYETHANOIC ACIDS

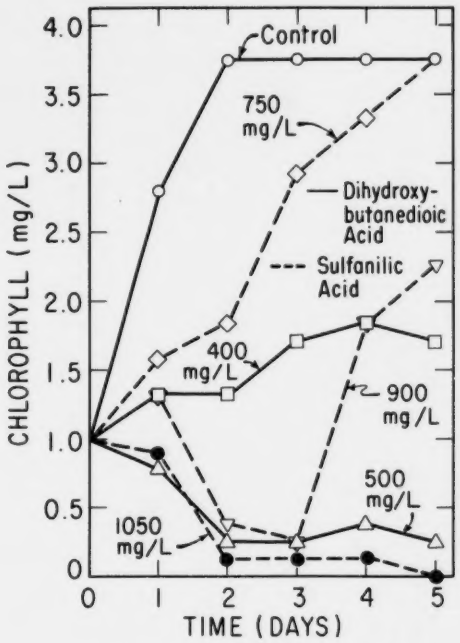

FIG. 4-18. EFFECTS OF 2, 3-DIHYDROXYBUTANEDIOIC AND SULFANILIC ACIDS

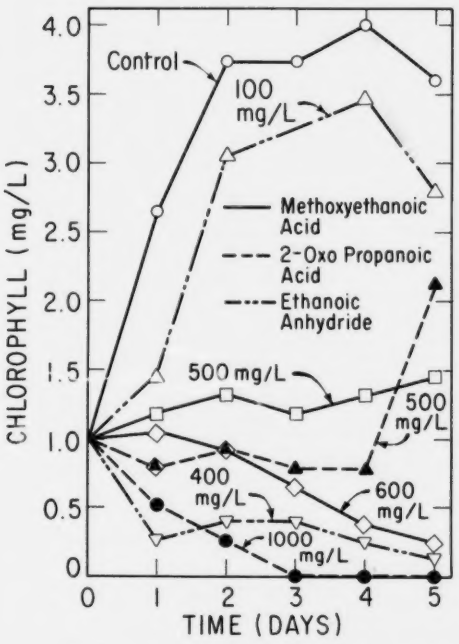

FIG. 4-19. EFFECTS OF METHOXY ETHANOIC ACID, 2-OXO PROPANOIC ACID AND ETHANOIC ANHYDRIDE

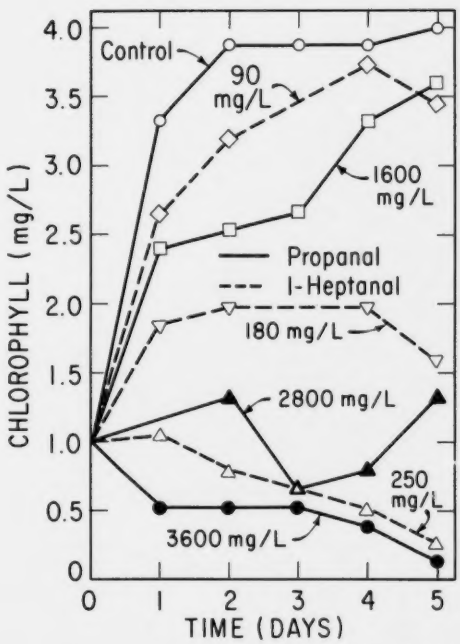

FIG. 4-20. EFFECTS OF PROPANAL AND HEPTANAL

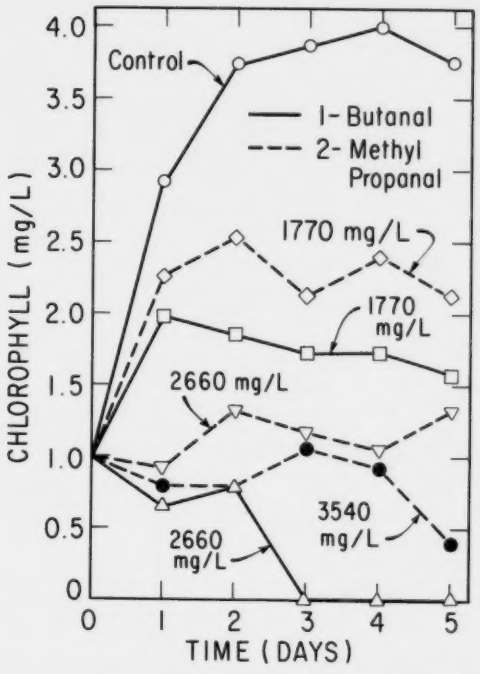

FIG. 4-21. EFFECTS OF 1-BUTANAL AND 2-METHYL PROPANAL

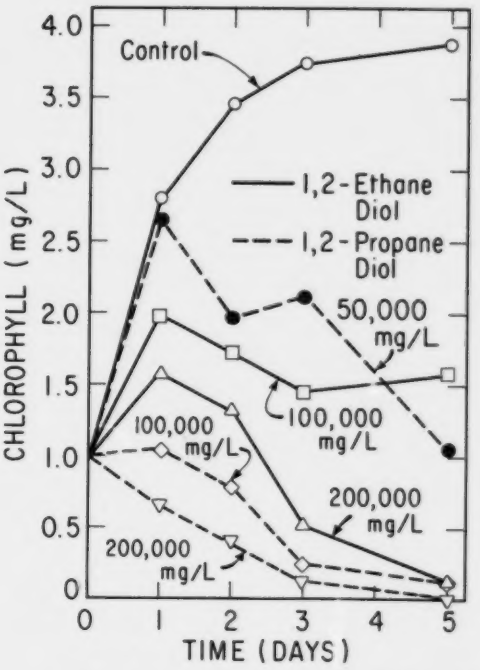

FIG. 4-22. EFFECTS OF GLYCOLS

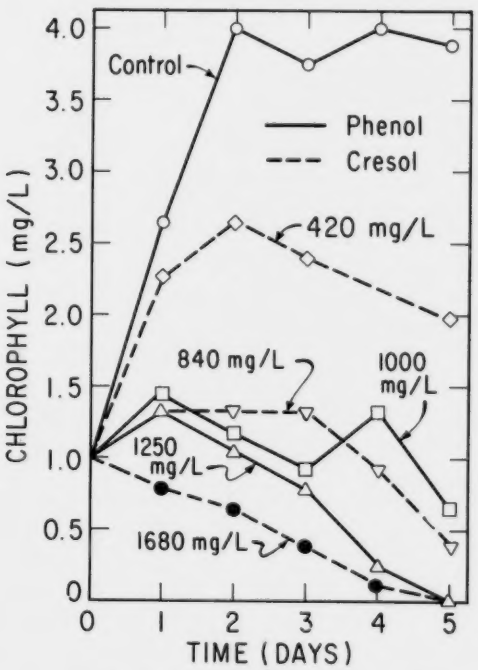

FIG. 4-23. EFFECTS OF PHENOL AND CRESOL

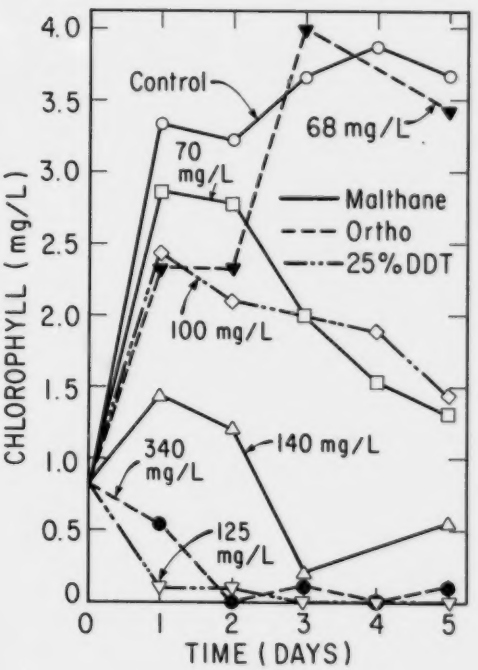

FIG. 4-24. EFFECTS OF COMMERCIAL PESTICIDES

volatility. The straight chain aldehyde with four carbon atoms in the molecule, 1-butanal, was more toxic than the corresponding branched chain aldehyde, 2-methyl propanal, Table 4-5 and Fig. 4-21. A similar relationship existed in the case of certain fatty acids and alcohols. At a concentration of 2,660 mg/L, 1-butanal reduced the chlorophyll content to zero by the third day and there was no recovery. Yet, at the same concentration of 2,660 mg/L of 2-methyl propanal, the chlorophyll content of algal cells remained well above 0.8 mg/L throughout the test period. Similar differences in chlorophyll content could be noticed at the concentrations of 1,770, 2,210 and 3,540 mg/L of the two aldehydes. 1-butanal and 2-methyl propanal were toxic to the algal species, respectively, at concentrations beginning at 2,660 mg/L and 3,540 mg/L.

The two glycols, 1,2-ethanediol (ethylene glycol) and 1,2-propanediol (propylene glycol), respectively, were toxic at the concentrations beginning at 200,000 mg/L and 100,000 mg/L, Table 4-6 and Fig. 4-22. These glycols were not significantly volatile, Table 3-1. At the concentration of 200,000 mg/L, 1,2-ethanediol did not eliminate the photosynthetic pigment during the initial 24 hours of contact. However, there was a gradual elimination of chlorophyll after one day. The results showed no evidence of recovery of chlorophyll production until the end of the test period.

At the concentration of 100,000 mg/L of 1,2-propanediol, the chlorophyll content of algal cells slightly increased during the initial 24-hour period. However, the test algae could not maintain the increasing trend of chlorophyll production after one day.

In fact, the concentration of the green pigment declined continuously until the fifth day. The results did not indicate any recovery in chlorophyll production.

Phenol and cresol, respectively, were toxic to algae at the concentrations beginning at 1,250 and 840 mg/L, Fig. 4-23 and Tables 4-7 and 4-8. The initial increase of chlorophyll synthesis by the algae for the first 24 hours and the steady decline of the synthesis during the rest of the test period was noted at a concentration of 1,250 mg/L of phenol. Similar trends were observed at certain concentrations of several organic compounds tested in this investigation. The glycols were two such examples. Cresol, at a concentration of 840 mg/L, showed the above-mentioned trends except that the chlorophyll production increased for the initial 48 hours instead of 24 hours which was the case for most other compounds.

The commercial pesticide, Malthane, at a concentration of 210 mg/L, eliminated the chlorophyll after three days of contact. This elimination continued until the end of the test period without any evidence of recovery. At a concentration of 140 mg/L of Malthane, a slight recovery in the synthesis of chlorophyll was noted between three and five days of contact, Fig. 4-24.

The commercial pesticide, Ortho, was toxic to algae at concentrations beginning at 340 mg/L. In the presence of 202 mg/L of Ortho, the test algae continued to synthesize the photosynthetic pigment throughout the test period and the final chlorophyll content was about 2.9 mg/L at the end of the test.

The pesticide, DDT, dissolved in the solvent, xylene, was effective in eliminating the green pigment when tested at a concentration of 125 mg/L, Fig. 4-24. An increase in chlorophyll formation for the initial 24-hour period and subsequent reduction in the concentration of the pigment during the rest of the test period was observed. Similar trends were typical of the two glycols, phenol, cresol and several other compounds.

Inactivation of Toxic Materials

It is known that some chemicals will materially reduce the toxicity of certain compounds to algae. To measure this effect, two sets of tests were undertaken with certain toxic substances. Two sets of test tubes were identical, except in one case an inactivator was added to the test tubes as well as the toxic chemical.

Toxic chemicals reduced the chlorophyll content when present above the toxic concentrations. A chemical was said to be an inactivator of toxicity when the addition of the inactivator to the toxic chemical increased the chlorophyll content instead of decreasing it.

The toxic effects of 2-thiouracil was reduced by adenine and uracil, Fig. 4-25. 2-thiouracil, at a concentration of 2,560 mg/L reduced the chlorophyll content to 0.4 mg/L at the end of the test period. However, the addition of 135 mg/L of adenine to the same concentration of 2-thiouracil increased the chlorophyll content to 3.74 mg/L in the same interval of time. The specific mechanism of the inactivation by adenine is not presently known (34).

Table 4-10. Inactivation of 2-thiouracil Toxicity by Uracil

2-thiouracil (mg/L)	Uracil (mg/L)	Transmittance (Per cent) After 1 day	After 2 Days	After 3 Days	After 5 Days	Final pH
Control	-	78	72	72	70	6.7
1,280	-	89	87	86	85	6.6
1,280	225	79	80	78	74	6.6
1,500	-	92	92	91	90	6.4
1,500	225	85	80	77	80	6.5
1,500	450	82	80	78	79	6.5
2,560	-	94	95	95	97	6.0
2,560	225	86	79	79	83	6.2

Table 4-11. Inactivation of 2-thiouracil Toxicity by Adenine

2-thiouracil (mg/L)	Adenine (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Control	-	77	73	73	72	71	6.5
-	135	79	74	74	75	75	6.6
-	400	80	75	75	77	77	6.5
1,280	-	91	88	87	86	83	6.5
1,280	135	79	70	70	69	65	6.5
1,280	400	80	71	72	68	65	6.6
2,560	-	94	95	95	95	97	6.0
2,560	135	76	72	73	72	72	6.2

At a concentration of 2,560 mg/L of 2-thiouracil, the chlorophyll content was reduced to 0.4 mg/L after five days of exposure; whereas, the addition of 225 mg/L of uracil to the same concentration of 2-thiouracil increased the chlorophyll production to 2.26 mg/L instead of decreasing it.

According to Steiner, et al., (48) many organic substances that are structurally similar to cellular building blocks like amino acids, purines, and pyrimidines can act as competitive inhibitors. The normal biosynthetic metabolism of several microorganisms is deranged by these competitive inhibitors. Several investigators showed that 2-thiouracil is a competitive inhibitor of cellular metabolism (35, 51).

Uracil and 2-thiouracil are structurally similar compounds. The toxicity of 2-thiouracil to algae was reduced in the presence of uracil. Hence it may be proposed that 2-thiouracil is a competitive inhibitor to Chlorella pyrenoidosa. In the absence of uracil, 2-thiouracil is incorporated in RNA and thereby 2-thiouracil acts as a toxic chemical to the test algae. This method of toxic action is suggested by several other investigators with respect to higher plants (34,48).

Orotic acid, cytidylic acid and hypoxanthine were not effective in reducing the toxicity of 2-thiouracil, Fig. 4-26. The reasons for such behavior cannot be clearly explained from the results of this study.

Phosphoglyceric acid (PGA) neutralized the toxic effects of ethanol, Fig. 4-27. Lefrancois and Quellet (28) suggested that ethanol interfered in the photosynthetic metabolism and suppressed

Table 4-12. Inactivation of 2-thiouracil Toxicity by Cytidylic Acid

2-thiouracil (mg/L)	Cytidylic Acid (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 5 Days	Final pH
Control	-	79	72	72	71	6.7
1,280	-	91	90	88	89	6.6
1,280	1,200	85	88	87	88	6.6
2,560	-	94	95	95	97	6.0
2,560	65	95	96	95	98	6.0
2,560	650	93	94	96	96	6.1

Table 4-13. Inactivation of 2-thiouracil Toxicity by Orotic Acid

2-thiouracil (mg/L)	Orotic Acid (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 3 Days	After 4 Days	After 5 Days	Final pH
Control	-	79	-	72	70	71	6.6
1,280	-	89	90	88	86	83	6.5
1,280	175	85	88	87	87	84	6.5
1,280	900	89	89	90	86	84	6.5
1,280	1,750	88	90	88	-	80	6.4
1,500	-	92	92	88	-	90	6.3
1,500	1,750	90	-	88	92	95	6.2
2,560	-	94	95	95	95	97	6.0
2,560	1,750	93	92	93	93	95	6.1

Table 4-14. Inactivation of 2-thiouracil Toxicity by Hypoxanthine

2-thiouracil (mg/L)	Hypoxanthine (mg/L)	Transmittance (Per cent) After 1 Day	After 2 Days	After 4 Days	After 5 Days	Final pH
Control	-	79	72	70	71	6.9
1,280	-	90	91	92	86	6.8
1,280	135	91	90	91	91	6.5
1,500	-	93	92	90	90	6.6
1,500	900	92	89	88	89	6.5
1,500	1,050	94	92	89	91	6.5
2,560	-	94	95	-	97	6.0
2,560	270	93	93	94	96	6.1
2,560	1,050	96	95	-	98	6.1

Table 4-15. Inactivation of Methanol Toxicity by Phosphoglyceric Acid (PGA)

Methanol (mg/L)	PGA (mg/L)	Transmittance (Per cent) After 1 Day	After 3 Days	After 4 Days	After 5 Days	Final pH
Control	-	77	70	71	70	6.9
40,000	-	83	82	81	87	6.7
40,000	1,600	82	81	80	87	6.7
50,000	-	87	91	93	97	6.0
50,000	400	88	90	93	96	6.1
50,000	800	86	92	95	98	6.0
60,000	-	92	95	96	98	6.0
60,000	400	93	95	97	98	5.9
60,000	1,600	94	93	-	98	6.0

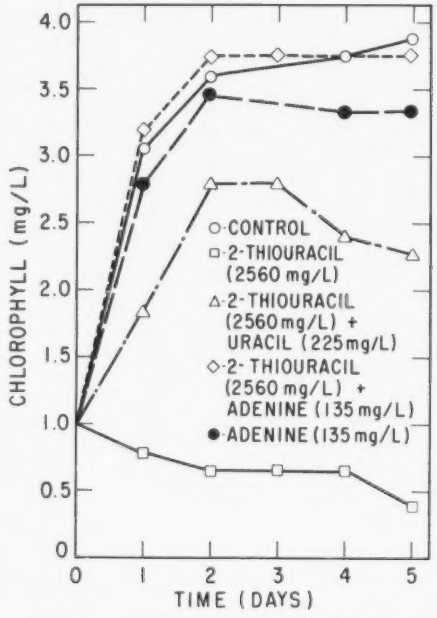

FIG. 4-25. INACTIVATION OF 2-THIOURACIL TOXICITY BY ADENINE AND URACIL

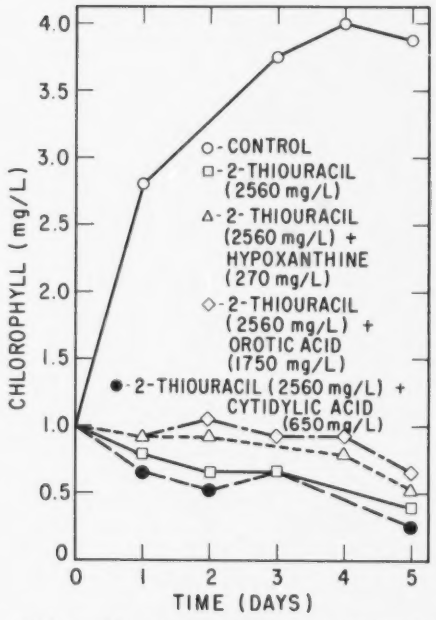
CHLOROPHYLL (mg/L)
TIME (DAYS)
○-CONTROL
□-2-THIOURACIL (2560 mg/L)
△-2-THIOURACIL (2560 mg/L) + HYPOXANTHINE (270 mg/L)
◇-2-THIOURACIL (2560 mg/L) + OROTIC ACID (1750 mg/L)
●-2-THIOURACIL (2560 mg/L) + CYTIDYLIC ACID (650 mg/L)

FIG. 4-26. INACTIVATION OF 2-THIOURACIL TOXICITY BY CYTIDYLIC ACID, OROTIC ACID AND HYPOXANTHINE

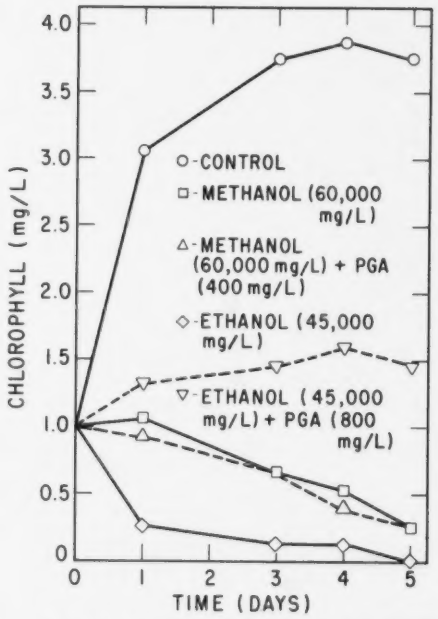

FIG. 4-27. INACTIVATION OF METHANOL AND ETHANOL TOXICITY BY PHOSPHOGLYCERIC ACID (PGA)

the formation of PGA. The results of the present study confirm the possibility that ethanol may interfere in the above-mentioned manner.

The addition of PGA to ethanol increased the chlorophyll production, whereas the presence of ethanol alone reduced the chlorophyll content. At a concentration of 45,000 mg/L of ethanol, the chlorophyll content of algal cells was reduced to zero after five days; whereas, the addition of 800 mg/L of PGA provided as much as 1.3 mg/L of chlorophyll. Similar results were noticed at other concentrations of ethanol and PGA.

The toxic behaviors of other alcohols cannot be generalized on the same basis. It was found that PGA could not inactivate the toxicity of methanol and 1-propanol, Figs. 4-27 and 4-28.

At a concentration of 60,000 mg/L, methanol reduced the chlorophyll content of the algal cells to 0.26 mg/L after five days of contact. When 400 mg/L and 1,600 mg/L, respectively, of PGA were introduced into the test tubes containing 60,000 mg/L of methanol, the chlorophyll content was still 0.32 and 0.26 mg/L after five days. Similarly, there was no apparent inactivation when the test tubes contained 40,000 mg/L of methanol and 1,600 mg/L of PGA.

The presence of 15,000 mg/L of 1-propanol reduced the chlorophyll concentration to 0.5 mg/L after five days. The introduction of 400 and 800 mg/L of PGA to the same concentration of propanol did not reestablish the chlorophyll level. Similar results were observed when different concentrations of PGA were added to the tubes containing 20,000 mg/L of 1-propanol.

Table 4-16. Inactivation of Ethanol Toxicity by Phosphoglyceric Acid (PGA)

Ethanol (mg/L)	PGA (mg/L)	Transmittance (Per cent) After 1 Day	After 3 Days	After 4 Days	After 5 Days	Final pH
Control	-	77	72	71	72	6.7
37,500	-	96	97	97	98	6.0
37,500	100	90	92	93	95	6.1
37,500	800	88	93	92	90	6.1
37,500	1,600	89	90	90	91	6.1
45,000	-	98	99	99	100	5.7
45,000	800	90	89	88	89	6.0
45,000	1,600	89	90	89	89	5.9

Table 4-17. Inactivation of 1-Propanol Toxicity by Phosphoglyceric Acid (PGA)

1-propanol (mg/L)	PGA (mg/L)	Transmittance (Per cent) After 1 Day	After 3 Days	After 4 Days	After 5 Days	Final pH
Control	-	77	70	70	70	6.9
15,000	-	91	88	90	96	6.0
15,000	400	92	89	91	95	6.0
15,000	800	90	87	90	94	6.1
20,000	-	99	96	97	98	5.9
20,000	400	98	97	98	99	5.9
20,000	1,600	97	96	96	95	6.0

There are two theories describing the possible reasons for the toxic behavior of o-phenanthroline. According to Lefrancois and Quellet (28), PGA could not be synthesized by Scenedesmus in the presence of o-phenanthroline. However, according to Fry and Pietrs (14), o-phenanthroline formed an iron complex with the reducing enzyme, pyridine nucleotide reductase. Hence, the toxic effect of o-phenanthroline should be neutralized either by PGA or by an increased dose of iron.

Two separate experiments were designed to investigate the applicability of the above-mentioned theories with respect to Chlorella pyrenoidosa. In the first experiment, PGA was used as a neutralizing chemical against the toxic effects of o-phenanthroline. PGA could not inactivate the toxic behavior of o-phenanthroline, Fig. 4-29. In the second experiment the concentration of iron was increased in the Knops solution by adding additional ferrous sulfate. It was found that the toxicity of o-phenanthroline was neutralized very effectively due to the presence of excess ferrous sulfate, Fig. 4-29. The excess available iron presumably satisfied the demands of o-phenanthroline to form an iron complex. Thus the reducing enzyme, pyridine nucleotide reductase, was probably unaffected by o-phenanthroline.

The presence of 30 mg/L of o-phenanthroline eliminated the chlorophyll by the third day. The presence of 1,600 mg/L of PGA, did not reduce the effect of o-phenanthroline.

At a concentration of 20 mg/L, o-phenanthroline reduced the chlorophyll content of the algae to 0.26 mg/L after five days. However, the addition of 1,000 mg/L of ferrous sulfate

Table 4-18. Inactivation of o-phenanthroline Toxicity by Phosphoglyceric Acid

o-phenanthroline (mg/L)	Phosphoglyceric Acid (mg/L)	Transmittance (Per cent) After 1 Day	After 3 Days	After 5 Days	Final pH
10	-	91	92	96	6.2
10	400	90	92	-	6.1
10	800	90	88	95	6.2
10	1,600	92	90	-	6.0
10	3,200	93	94	97	6.3
20	-	94	96	98	6.2
20	400	92	97	99	6.1
20	800	93	98	97	6.2
20	1,600	-	-	-	-
20	2,400	95	98	99	-
30	-	99	100	100	5.9
30	400	98	100	100	5.8
30	800	98	100	100	5.9
30	1,600	97	100	100	6.0
Control	-	77	73	70	6.5

Table 4-19. Inactivation of o-phenanthroline Toxicity by Ferrous Sulfate

o-phenanthroline (mg/L)	Ferrous-Sulfate (mg/L)	Transmittance (Per cent) After 1 Day	After 4 Days	After 5 Days	Final pH
10	-	92	95	96	6.2
10	500	80	70	71	6.3
10	1,000	85	76	74	6.5
10	3,000	-	80	-	-
20	-	94	96	97	6.2
20	500	81	70	68	6.5
20	1,000	87	70	69	6.4
0	2,000	78	71	71	6.6
0	500	80	72	73	6.5
Control	-	79	70	70	6.5

Table 4-20. Inactivation of CCCP Toxicity by Fructose-1,6-Diphosphate

CCCP (mg/L)	Fructose-1,6-Diphosphate (mg/L)	Transmittance (Per cent) After 1 Day	After 3 Days	After 5 Days	Final pH
Control	-	79	70	70	6.8
5.0	-	86	84	82	6.6
5.0	300	87	85	84	6.5
5.0	600	84	82	81	6.5
5.0	1,500	87	86	85	6.5
12.5	-	89	99	100	6.2
12.5	300	90	99	100	6.1
12.5	600	91	99	100	6.2
12.5	1,500	88	98	100	6.0
50.0	-	96	100	100	5.9
50.0	300	95	100	100	6.0
50.0	600	97	100	100	5.9
50.0	1,500	98	100	100	5.9

to 20 mg/L of o-phenanthroline increased the chlorophyll production to 3.46 mg/L. Such significant neutralization effects were noticed under various concentrations of ferrous sulfate.

The toxic effects exhibited by m-chlorocarbonyl cyanide phenylhydrazone (CCCP) were not neutralized by the addition of fructose-1,6-diphosphate, Fig. 4-30. Bamberger, et al., (5) showed that the inhibition of spinach chloroplasts by CCCP could be overcome by the addition of fructose-1, 6-diphosphate. However, the present experiment with Chlorella pyrenoidosa did not show any such inactivation effect.

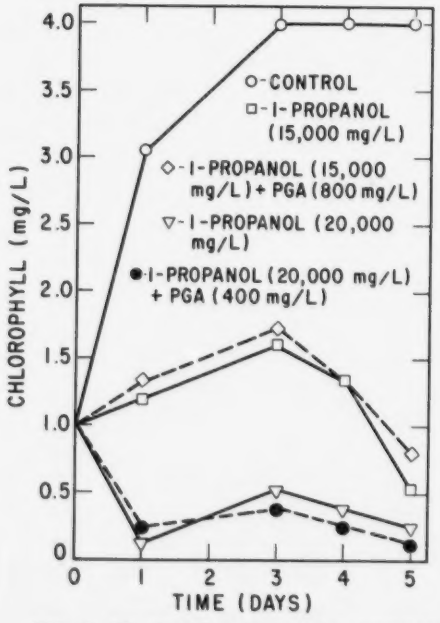

FIG. 4-28. INACTIVATION OF I-PROPANOL TOXICITY BY PHOSPHOGLYCERIC ACID (PGA)

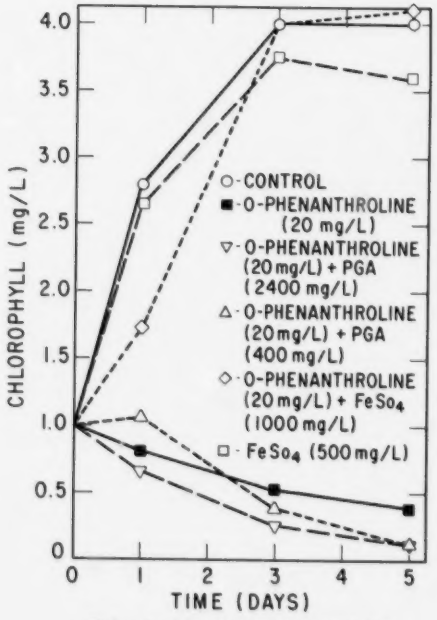
CHLOROPHYLL (mg/L)
TIME (DAYS)
O-CONTROL
■-O-PHENANTHROLINE (20 mg/L)
▽-O-PHENANTHROLINE (20 mg/L) + PGA (2400 mg/L)
△-O-PHENANTHROLINE (20 mg/L) + PGA (400 mg/L)
◇-O-PHENANTHROLINE (20 mg/L) + FeSo4 (1000 mg/L)
□-FeSo4 (500 mg/L)

FIG. 4-29. INACTIVATION OF O-PHENANTHROLINE TOXICITY BY PHOSPHOGLYCERIC ACID (PGA) AND FERROUS SULFATE ($FeSo_4$)

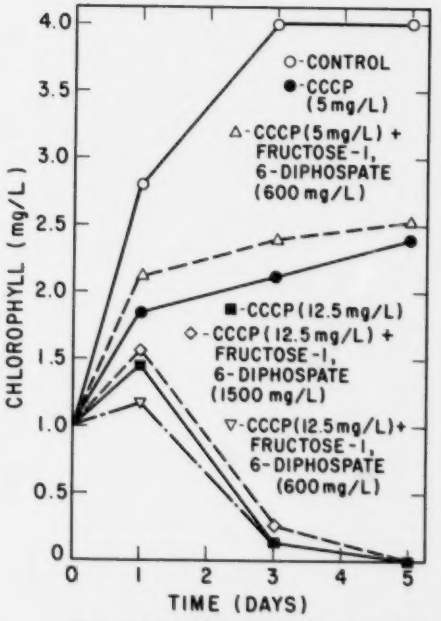
CHLOROPHYLL (mg/L)
TIME (DAYS)
O-CONTROL
●-CCCP (5 mg/L)
△-CCCP (5 mg/L) + FRUCTOSE-1, 6-DIPHOSPATE (600 mg/L)
■-CCCP (12.5 mg/L)
◇-CCCP (12.5 mg/L) + FRUCTOSE-1, 6-DIPHOSPATE (1500 mg/L)
▽-CCCP (12.5 mg/L) + FRUCTOSE-1, 6-DIPHOSPATE (600 mg/L)

FIG. 4-30. INACTIVATION OF CCCP TOXICITY BY FRUCTOSE-1, 6-DIPHOSPATE

CHAPTER V

DISCUSSION

The assimilative capacity of waste stabilization ponds is affected when the photosynthetic capabilities of algae are decreased. This discussion considers possible modifications to design criteria, relationship of molecular structure to toxicity, and various modes of interaction.

Design Modifications

The toxicity of a chemical to typical microorganisms in aquatic environments depends on various factors such as the type and growth habits of the organisms, the concentration and molecular structure of the chemical and the environmental conditions. Neither these factors nor their interrelationships are well-defined. Hence, it is practically impossible to simulate field conditions in the laboratory. Consequently in a laboratory test program it is necessary to limit the interacting variables as much as possible.

The results of this investigation can be used to improve existing design equations, particularly when certain industrially produced organic wastes are present. Because most design relationships are based on the treatment of domestic wastes, the ponds receiving industrial wastes are likely to be under-designed.

Hermann and Gloyna (23) and as modified by Gloyna (19) suggested a relationship for the design of domestic ponds. This concept may be used as a basis for industrial pond design. Whereas the earlier designs were based on a depth of 3.5 feet, it

is now recognized that a depth of six feet is more desirable. Consequently, for domestic-like waste streams an acceptable design equation might be as follows:

a. Using the original Hermann-Gloyna equation,

$$V = 5.37 \times 10^{-8} \, N_q Y \, 1.072^{(35-T)} \quad \text{(Depth = 3.5 feet)} \qquad \text{Eq. 1}$$

where, V = Volume of the Pond in acre-feet,
N_q = Flow of Wastewater in gal/day,
Y = Influent B.O.D. in mg/L,
and T = Temperature in °C.

b. Using the modified version,

$$V = 9.2 \times 10^{-8} \, N_q Y \, 1.072^{(35-T)} \quad \text{(Depth = 6.0 feet)} \qquad \text{Eq. 2}$$

Now, for waste streams which contain industrially produced organics, a further modification is suggested to Equation 1:

$$V = 9.2 \times 10^{-8} \, N_q Y \, 1.072^{(35-T)_K} \qquad \text{Eq. 3}$$

where, K = Toxicity coefficent.

The value of this coefficient will depend on the type and the concentration of the toxic organic chemical as well as its susceptibility to biodegradation by bacteria. The latter effect although very important was not included within the scope of this study. Therefore, the equation is believed to provide a conservative design criteria.

Heptanol, at a concentration of 525 mg/L, reduced the chlorophyll content of the algal cells from 1.0 mg/L to 0.53 mg/L after five days of contact. Thus chlorophyll was reduced to about 50 per cent of its initial value. For illustrative purposes and disregarding bacterial reduction of heptanol, the assimilative capacity of an aerobic or perhaps even facultative waste stabilization pond is related to the total available chlorophyll. Hence the volume of the waste stabilization pond

should be doubled to maintain the desired treatment efficiency of the pond when the pond influent contains 525 mg/L of heptanol. Consequently, the corresponding value of K is suggested as 2.0.

For many chemicals investigated in this study, the toxic concentration which would have reduced 50 per cent of chlorophyll in five days was calculated by mathematical interpolation. Such interpolation is necessary because it was not possible in the actual experiments to arrive at the 50 per cent chlorophyll reduction value for a given concentration of the toxic chemical. As an example, at a concentration of 300 mg/L of hexanoic acid, the chlorophyll was reduced from 1.0 mg/L to 0.79 mg/L. At the next higher concentration, namely, 350 mg/L of hexanoic acid, the chlorophyll was completely eliminated. Hence, the minimum toxic concentration value of the organic acid is assumed to be between 300 and 350 mg/L and calculated by interpolation as 320 mg/L. Thus, the value of K is suggested as 2.0 when the concentration of hexanoic acid is 320 mg/L. Similarly, the toxic concentrations of other chemicals which would have eliminated 50 per cent of the chlorophyll are calculated and shown in Table 5-1. This table also shows the concentrations of the organic chemicals and the corresponding chlorophyll content of the test alga after five days of contact.

Limitations of K Value

Certain limitations should be considered in using these toxicity coefficients. The laboratory model was different from a waste stabilization pond in several respects. Hence, these toxicity values and coefficients are relative. The toxicity values

Table 5-1. Calculated Values of the Toxic Concentrations.*

Organic Chemical	Concentration (mg/L)	Chlorophyll Content of Algal Cells After Five Days (mg/L)	Calculated Values of the Toxic Concentration (mg/L)
Methanoic Acid	180	1.72	220
	225	0.26	
Ethanoic Acid	320	1.32	350
	365	0.0	
Propanoic Acid	230	0.92	250
	276	0.0	
Butanoic Acid	325	1.05	340
	350	0.0	
2-methyl propanoic Acid	325	1.98	345
	350	0.0	
Pentanoic Acid	250	1.32	280
	300	0.0	
3-methyl butanoic Acid	300	2.26	400
	400	0.53	
Hexanoic Acid	300	0.79	320
	350	0.0	
Heptanoic Acid	150	1.32	180
	200	0.0	
Octanoic Acid	200	0.79	220
	250	0.0	

Table 5-1 (continued)

Organic Chemical	Concentration (mg/L)	Chlorophyll Content of Algal Cells After Five Days (mg/L)	Calculated Values of the Toxic Concentration (mg/L)
Ethanedioic Acid	200	2.66	290
	300	0.26	
Propanedioic Acid	400	0.66	460
	500	0.40	
Butanedioic Acid	2,000	0.79	2,200
	2,500	0.0	
Pentanedioic Acid	1,000	0.92	1,200
	1,500	0.0	
Hexanedioic Acid	750	0.79	900
	1,000	0.0	
Heptanedioic Acid	500	2.93	700
	750	0.26	
Methanol	26,000	1.85	31,100
	32,500	0.13	
Ethanol	23,100	1.72	27,200
	28,900	0.0	
1-propanol	7,900	1.85	11,200
	11,800	0.26	
2-propanol	15,600	0.92	17,400
	19,500	0.0	

Table 5-1 (continued)

Organic Chemical	Concentration (mg/L)	Chlorophyll Content of Algal Cells After Five Days (mg/L)	Calculated Values of the Toxic Concentration (mg/L)
1-butanol	8,000	0.66	8,500
	10,000	0.0	
2-butanol	8,000	0.92	8,900
	10,000	0.0	
3-butanol	20,000	2.42	24,200
	25,000	0.13	
1-hexanol	1,200	1.05	1,275
	1,350	0.0	
2-hexanol	2,400	3.2	3,100
	3,200	0.26	
Heptanol	450	1.85	525
	525	0.53	
Octanol	200	0.92	250
	250	0.53	
Propenoic Acid	100	0.79	120
	150	0.0	
Butenoic Acid	250	1.45	280
	300	0.0	
2,3-dihydroxy butanedioic Acid	400	1.72	480
	500	0.26	
Hydroxyethanoic Acid	2,500	0.66	2,700
	3,000	0.26	

Table 5-1 (continued)

Organic Chemical	Concentration (mg/L)	Chlorophyll Content of Algal Cells After Five Days (mg/L)	Calculated Values of the Toxic Concentration (mg/L)
Sulfanilic Acid	900	0.92	
	1,050	0.0	970
Methoxyethanoic Acid	500	1.45	
	600	0.26	580
2-oxo propanoic Acid	500	2.12	
	1,000	0.0	880
Ethanoic Anhydride	300	1.05	
	400	0.13	360
Propanal	3,200	1.05	
	3,600	0.13	3,450
1-butanal	2,200	1.45	
	2,650	0.0	2,500
2-methyl propanal	2,660	1.32	
	3,540	0.4	3,450
1-heptanal	180	1.58	
	250	0.26	240
1,2-ethanediol	150,000	1.32	
	200,000	0.13	180,000
1,2-propanediol	50,000	2.40	
	100,000	0.13	92,000
Phenol	1,000	0.66	
	1,250	0.0	1,060

Table 5-1 (continued)

Organic Chemical	Concentration (mg/L)	Chlorophyll Content of Algal Cells After Five Days (mg/L)	Calculated Values of the Toxic Concentration (mg/L)
Cresol	420	1.98	800
	840	0.4	
Malthane	140	0.66	160
	210	0.0	
Ortho (Pesticide)	200	2.93	320
	340	0.13	
DDT in Xylene	100	1.72	120
	125	0.0	

* Based on 50 per cent chlorophyll reduction.
Hence, the value of K will be 2.0.
Calculated chlorophyll content after five days is equal to 0.5 mg/L.

are low values and the coefficients are high values, each representing conservative values from a design standpoint. The availability of excess nutrients may influence the toxicity values. For example, it was demonstrated that a common industrial pollutant such as ferrous sulfate (500 to 2,000 mg/L) may reduce the toxicity of o-phenanthroline. However, it must be recalled that ferrous sulfate was one of the inorganic nutrients used in algal growth media.

Bacterial Reduction Effect

The biodegradation of many organic pollutants will most certainly decrease the values of K. Little work has been done to measure the breakdown of specific organics by bacteria in waste stabilization ponds. Hence, a discussion of experiments in which the organic materials were degraded by the bacteria in streams and activated sludge units is presented.

Ludzack and Ettinger (29) have gathered available information on the degradation of certain organic chemicals under aerobic conditions. Depending on test conditions and concentrations of the organic chemicals, as much as 90 per cent and as low as three per cent of certain alcohols, short chain fatty acids and aldehydes were aerobically degraded in activated sludge units and certain streams (36,7).

Pipes and Jones (40) showed that Sphaerotilus can decompose fatty acids and alcohols very effectively, whereas the dibasic acids cannot be degraded. Randall (41) suggested that the phosphorylated pesticide, malathion, can be dissipated in water under turbulent conditions and that certain aerobes can degrade

it. According to Randall, up to one mg of malathion can be utilized as a source of energy by 15 mg of microbial solids. Another author reports that about 42 per cent of phenol was removed in an activated sludge plant in one day when the concentration of phenol was 40 mg/L in the influent (7). However, when the concentration was increased to 200 mg/L, only eight per cent of the chemical was removed.

Engelbrecht and McKinney (7) investigated the biodegradation of pure organic chemicals using activated sludge cultures. A concentration of 333 mg/L of pentanoic acid in the influent was reduced by 99 per cent after one day. Similar results were obtained for hexanoic and heptanoic acids.

Although biodegradation will occur as a result of bacterial activity, industrial shock loads may seriously affect the aerobic system of a large pond. Ideal conditions prevailed in the laboratory for the ideal growth of the test algae. Thus, a given concentration of an organic waste may exhibit a greater toxic effect in the ponds than in the laboratory.

A toxic material which destroyed 50 per cent of the chlorophyll content of Chlorella pyrenoidosa may be more or less toxic to other groups of algae. According to one report (1), trimethylalkyl ammonium chloride, at a concentration of two mg/L, was algicidal to all the blue-green algae tested and was algistatic to all but two species of green algae tested.

As shown above, field conditions may decrease the value of K due to bacterial action and increase the value due to non-ideal environment. As a concluding remark, it is suggested that

a toxicity correction be applied in design practices.

Toxicity-Structure Relationship

The toxicity values of short chain fatty acids, dicarboxylic acids, alcohols and aldehydes showed that the molecular structure and number of carbon atoms in the molecules of these chemicals were important in determining the toxic characteristics. Several factors make it difficult to explain the simple rules which describe toxicity strictly as a function of structure. Relatively very little work has been done in the area of toxicology using algae as the test organism. Malina (31) and Gloyna and Malina (20) reviewed the literature related to the toxicity of petrochemicals in aquatic environments and indicated that the majority of data on the toxic effects of organic chemicals deal with fish as the test animal.

Stressing the importance of developing new biochemical-toxicological laws, George (15) suggested that the possibility of discovering a new law is remote. Even if a general relationship or a law is suggested for the toxicity of certain structurally similar compounds, it might be difficult to explain the specific mechanisms of toxic actions. Stokinger (49) further suggests that the side reactions and products further complicate the problem to a point where easily understood rules cannot be developed.

Chemically or biochemically induced changes may result in differences between the original toxic compound introduced in a toxicity test and the actual material interfering in the metabolic pathway of the chemicals. According to Ludzack and

Ettinger (29), two examples in which such changes occur are the unsaturated compounds and nitriles.

According to the present investigation, the higher molecular weight alcohols were more toxic to algae than the lower members. There is evidence of such behavior in the literature, but no definite explanation has been offered (28). It cannot be generalized for all chemicals that toxicity increases with increasing molecular weight. The toxicity of dicarboxylic acids decreased to a minimum and then increased with increasing numbers of carbon atoms. The short chain fatty acids with odd number of carbon atoms were more toxic than the corresponding next acids with even number of carbon atoms. The specific reasons for such relationships are not known.

The effects of branching on biological resistance are important in certain acids, alcohols and aldehydes. The arrangement of carbon atoms in straight chains or branched chains was significant in exhibiting the toxic effects of these compounds. Certain straight chain acids, alcohols and aldehydes were more toxic than the corresponding branched chain chemicals.

Modes of Interaction

Toxic effects become apparent through different modes of interaction. For example, these interactions may be between the toxic chemical and one of the metabolic intermediates or the enzyme system. At least three such pathways are suggested from the results of this investigation:

First, a toxic chemical can suppress the formation of one of the important metabolic intermediates of photosynthesis, like

phosphoglyceric acid (PGA). Thus, the chemical interferes in the photosynthetic metabolism and thereby reduces the algal growth. Ethanol is an example of such a chemical group. Ethanol, at relatively high concentration (above 29,000 mg/L), suppressed the formation of PGA.

Second, certain organic chemicals can act on the enzymes necessary for the photosynthetic metabolism and thereby deplete some elements from the enzymes. Thus, as a result of the interaction between the toxic chemical and the enzyme, the affected enzyme cannot carry on the normal metabolism of the algal cells. The chelating agent, o-phenanthroline, is an example of such toxic organic substances. o-phenanthroline interferes in the normal activity of the reducing enzyme, pyridine nucleotide reductase. Yet, the presence of excess available ferrous sulfate (500 to 2,000 mg/L), one of the inorganic nutrients, completely neutralized the toxicity of o-phenanthroline. Thus, the availability of an excess of certain inorganic nutrients in the waste stabilization ponds may reduce the toxicity of some organic chemicals.

Third, certain toxic organic materials compete with the cellular building blocks like amino acids, pyrimidine derivatives and vitamins,because they are structurally similar to these building blocks. Thus the normal metabolsim of the exposed cells is affected. 2-thiouracil is such an example for it interferes with algal metabolism. 2-thiouracil competes with uracil to be fixed into RNA. Thus, the synthesis of RNA is affected in the presence of 2-thiouracil. This mode of action of the toxic chemicals is

generally termed competitive inhibition.

Some of the organic chemicals like 2-thiouracil may not be present in most of the industrial wastes. However, they exhibit typical toxic characteristics and represent one of the important groups of toxic chemicals. The understanding of the toxic behaviors of such chemicals as 2-thiouracil is necessary and important. Such results may lead to a better understanding of the treatability of more commonly present chemicals in the industrial wastes.

APPENDIX I

Nutrient Solution

Dissolve 0.50 gram of purified ethylene diamine tetra acetic acid (EDTA) in 800 ml of boiling water and cool the contents.

Add the stock solutions of the macroelements and microelements as shown in the Tables I-1 and I-2.

Adjust the pH to 7.2 by adding KOH as required.

Table I-1. Macroelements of Knops Solution

Salt	Molecular Weight	Stock Solution		Final Medium Concentration (gm/L)
		Concentration (gm/L)	Use (ml/L)	
$MgSo_4.7H_2O$	246.5	50.0	50.0	2.50
KNo_3	101.1	25.0	50.0	1.25
KH_2Po_4	136.1	25.0	20.0	0.50

Table I-2. Microelements of Knops Solution

Salt	Molecular Weight	Stock Solution		Final Medium Concentration (Elemental mg/L)
		Concentration (gm/L)	Use (ml/L)	
$Ca\ Cl_2$	111.0	8.35	10.0	30
H_3Bo_3	61.8	11.42	10.0	20
$FeSo_4.7H_2O$	278.0	4.98	10.0	10
$ZnSo_4.7H_2O$	288.0	8.82	10.0	20
$Mn\ Cl_2 14H_2O$	198.0	1.44	10.0	4
$Mo\ O_3$	144.0	0.71	10.0	4
$Cu\ So_4.5H_2O$	250.0	1.57	10.0	4
$Co(No_3).6H_2O$	291.0	0.49	10.0	1

APPENDIX II

Chlorophyll Extraction

Step 1.

Transfer with a pipette 2.5 ml of algal suspension into a centrifuge tube. Centrifuge for 15 minutes at 3,400 rpm, 9.5 inch diameter head. The centrifugal force required to deposit the cells of Chlorella pyrenoidosa is about 1,500 dynes.

After centrifuging, decant the supernatant thoroughly; add distilled water, suspend and wash algal residue; centrifuge again for 15 minutes at 3,400 rpm. After second centrifuging, decant the supernatant, leaving approximately 0.25 ml of water and algal cells.

Step 2.

Suspend the cells by rapidly thumping centrifuge tube with forefinger. Do not stir with stirring rod; be careful to maintain a firm grip on lip of centrifuge tube with thumb and forefinger of the other hand.

Coagulate suspended algae by heating in a boiling water bath for 30 to 45 seconds. The actual time will depend on the amount of water left in the centrifuge tube.

Step 3.

Add 2.0 to 2.5 ml of methanol and stir, removing hardened algae from sides of centrifuge tube. Heat in water bath for 15 seconds. The tube should be partially submerged, withdrawn and shaken periodically. Permit the contents to cool for at least five minutes before proceeding further.

Heat in boiling water bath with constant shaking. Do not

permit the contents to boil over. Continue until approximately 0.5 ml of the extract remains. Centrifuge for 15 minutes at 3,400 rpm and filter extract through medium sintered glass filter. Wash lip of centrifuge tube and filter with not more than 3 ml of 80 per cent acetone-water solution.

Step 4.

Repeat Step 3.

For complete removal of color, wash sides of centrifuge tube and suspend cells with 1.0 ml of 80 per cent acetone-water solution. Centrifuge for 15 minutes at 3,400 rpm and filter the supernatant. Wash lip of centrifuge tube and filter with not more than 1.5 ml of 80 per cent acetone-water solution.

Transfer filtered extract to 10 ml graduate cylinder, wash centrifuge tube with not more than 0.5 ml of 80 per cent acetone-water solution and later bring final volume to 10.0 ml.

The chlorophyll content is determined by use of Model B Beckman Spectrophotometer, red filter, and 652 mμ wave length setting. For best results the extract must not be stored for more than 12 hours. In all cases the contents must be secured to prevent evaporation and stored in a dark place.

APPENDIX III

Effects of Bacteria on Test Results

Special tests were conducted to make sure that there was no gross bacterial effects. The results of these tests are shown in Table III-1. Also, the test data for the algal growth in the presence of bacteria and other microorganisms are given in Table III-2.

Table III-1. Effects of Bacteria on Algal Suspension

Date	Test Chemical	Concentration of the Chemical (mg/L)	Type of Suspension	Result
2-3-1964	Formic Acid	50	Slightly Turbid	Slight Effects
		100	Clear	No Gross Effects
		200	Clear	No Gross Effects
		250	Clear	No Gross Effects
	Control	-	Clear	No Gross Effects
5-6-1964	Succinic Acid	2,000	Clear	No Gross Effects
		2,500	Clear	No Gross Effects
		3,000	Clear	No Gross Effects
		3,000	Clear	No Gross Effects
	Control	-	Clear	No Gross Effects
6-15-1964	Propanal	3,500	Clear	No Effects
		4,000	Clear	No Effects
		4,500	Slightly Turbid	Slight Effects
	Control	-	Clear	No Effects
7-17-1964	Ethanol	15,000	Clear	No Effects
		30,000	Slightly Turbid	Slight Effects
		37,500	Clear	No Effects
		45,000	Clear	No Effects
		60,000	Clear	No Effects
	Control	-	Clear	No Effects

Table III-1 (continued)

Date	Test Chemical	Concentration of the Chemical (mg/L)	Type of Suspension	Result
9-8-1964	Hexanol	750	Clear	No Effects
		900	Clear	No Effects
		1,050	Clear	No Effects
		1,200	Clear	No Effects
		1,350	Clear	No Effects
	Control	-	Clear	No Effects

Table III-2. Symbiotic Effects: Bacterial Growth

Chemical Tested	Concentration (mg/L)	Transmittance (Per Cent) After 1 Day	Transmittance (Per Cent) After 5 Days	Remarks
Control	-	84	71	Sterile Glassware, Aseptic Technique
Methanoic Acid	100	85	83	
	200	95	98	
1-Propanol	10,000	94	86	
	15,000	95	98	
	20,000	100	100	
2-Propanol	10,000	90	81	
	15,000	92	88	
	20,000	93	93	
	25,000	94	100	
Control	-	84	70	Without Sterilization, without using Aseptic Techniques
Methanoic Acid	100	86	82	
	200	97	99	
1-propanol	10,000	93	85	
	15,000	97	99	
	20,000	99	99	
2-propanol	10,000	89	80	
	15,000	93	89	
	20,000	93	94	
	25,000	94	99	

APPENDIX IV

Standard Chlorophyll Curve

A standard curve was prepared. Duplicate samples of an algal suspension each containing 2.5 ml were used. The final extract was diluted to develop the standard curve.

The chlorophyll concentration in mg/L was calculated from the equations developed by Arnon (3) and Mackinney (30) as shown below:

$$C_a = 12.7 \times D_{663} - 2.69 \times D_{645}$$

$$C_b = 22.9 \times D_{645} - 4.68 \times D_{663}$$

$$C_{a+b} = \frac{D_{652}}{0.0345} \text{ mg/L}$$

where, D = optical density at respective wavelengths
C_a = chlorophyll 'a' concentration in mg/L
C_b = chlorophyll 'b' concentration in mg/L
C_{a+b} = concentration of chlorophylls 'a' + 'b'

To provide a rough check for the determination of total chlorphyll, the light absorption data for chlorophyll 'a' and 'b' were plotted by Arnon (3). The curves were found to intersect at a wavelength of 652 mμ. Hence a wavelength of 652 mμ is the most optimum because various proportions of chlorophyll 'a' and 'b' least affect the results.

The optical density of the chlorophyll extract was equal to 0.1410 at a wavelength of 652 mμ. Thus, the chlorophyll concentration of the algal sample was,

$$= \frac{D_{652}}{0.0345} \text{ mg/L}$$

$$= \frac{0.1410}{0.0345} = 4.04 \text{ mg/L}$$

For a mass of algal cells having a concentration of 1.00

gm/L, the chlorophyll content was 4.04 mg/L. Table IV-1 shows the data used in preparing the standard curve.

Table IV-1. Data for the Standard Curve

Weight of Algal Cells (gm/L)	Transmittance (Per cent) (T)			Optical Density	Chlorophyll Concentration of Algal Cells (mg/L)
	Sample (1) (T_1)	Sample (2) (T_2)	Average (T)		
1.008	72.0	72.5	72.3	0.1410	4.0
0.807	78.0	77.0	77.5	0.110	3.2
0.667	81.0	80.5	80.8	0.093	2.67
0.504	84.5	85.5	85.0	0.070	2.0
0.334	90.0	90.5	90.3	0.044	1.33
0.252	92.0	92.5	92.3	0.0350	1.0
0.167	94.5	95.0	94.7	0.0240	0.66
0.126	95.5	96.5	96.0	0.0177	0.50

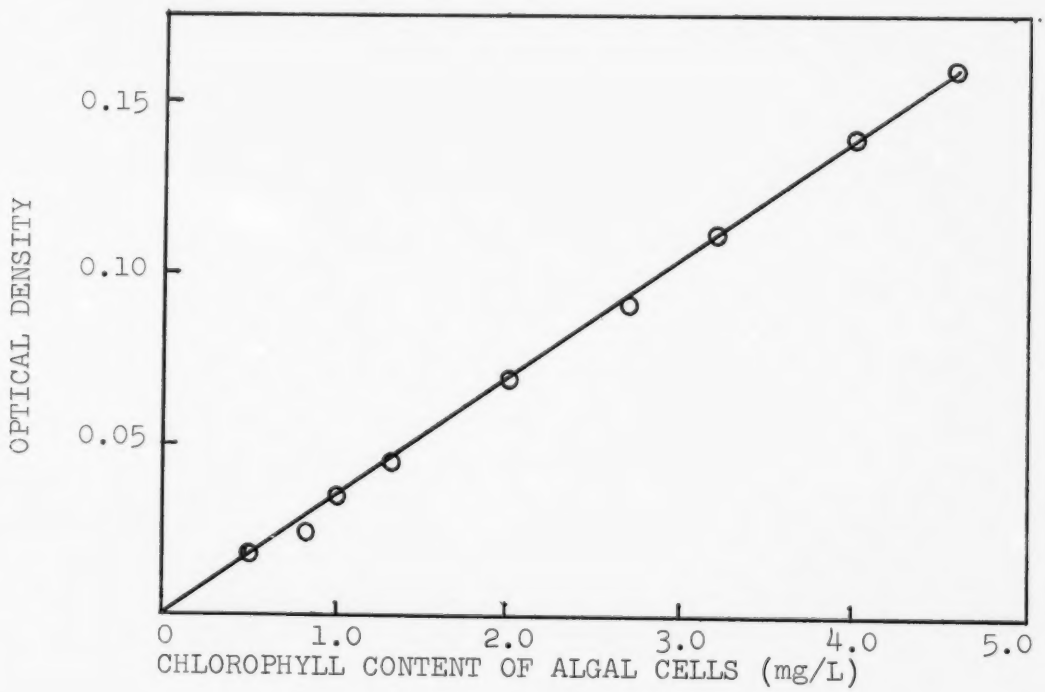

FIG. IV-1. STANDARD CHLOROPHYLL CURVE

APPENDIX V

Physical Constants

The properties of the chemicals which were of importance in selecting the organic chemicals for this study were solubility in water, specific gravity and boiling point. These physical constants are published in The Handbook of Chemistry and Physics (24) and are presented in Table V-1. The names recommended by the International Union of Chemistry (IUC) are used for the chemicals. The ingredients of the commercial pesticides are given in Table V-2.

Table V-1. Physical Constants

Organic Compound	Formula	Molecular Weight	Boiling Point (°C)	Specific Gravity	Solubility (gms/100 ml of water)
Fatty Acids					
Methanoic Acid	$HCOOH$	46	100	1.23	∞
Ethanoic Acid	CH_3COOH	60	118	1.05	∞
Propanoic Acid	CH_3CH_2COOH	74	141	0.99	∞
Butanoic Acid	$CH_3(CH_2)_2COOH$	88	163	0.96	∞
2-methyl propanoic Acid	$(CH_3)_2CH\ COOH$	88	154	0.95	20
Pentanoic Acid	$CH_3(CH_2)_3COOH$	102	187	0.944	∞
3-methyl butanoic Acid	$(CH_3)_2CHCH_2COOH$	102	176	0.940	4.2
Hexanoic Acid	$CH_3(CH_2)_4COOH$	116	205	0.945	C.4
Heptanoic Acid	$CH_3(CH_2)_5COOH$	130	223	0.913	S
Octanoic Acid	$CH_3(CH_2)_6COOH$	144	237	0.910	0.25
Di-carboxylic Acids					
Ethanedioic Acid	$(COOH)_2.2H_2O$	126	Subl	1.65	9.5

Table V-1 (continued)

Organic Compound	Formula	Molecular Weight	Boiling Point (°C)	Specific Gravity	Solubility (gms/100 ml of water)
Propanedioic Acid	HOOC CH_2COOH	104	d	1.63	73.5
Butanedioic (succinic)	HOOC$(CH_2)_2$COOH	118	235d	1.56	6.8
Pentanedioic Acid	HOOC$(CH_2)_3$COOH	132	304d	1.43	64.0
Hexanedioic Acid	HOOC$(CH_2)_4$COOH	146	265	1.37	1.5
Heptanedioic Acid	HOOC$(CH_2)_5$COOH	160	272	1.33	5.0
Alcohols					
Methanol	CH_3OH	32	64	0.796	∞
Ethanol	CH_3CH_2OH	46	78	0.790	∞
1-propanol	$CH_3(CH_2)_2OH$	60	97	0.800	∞
2-propanol	$CH_3CHOH\ CH_3$	60	82	0.785	∞
1-butanol	$CH_3(CH_2)_3OH$	74	117	0.810	7.9
2-butanol	$(CH_3)_2CHCH_2OH$	74	108	0.80	9.5
3-butanol	$(CH_3)_3COH$	74	83	0.79	∞
1-hexanol	$CH_3(CH_2)_4CH\ OH$	102	157	0.82	9.59
2-hexanol	$CH_3CHOH(CH_2)_3CH_3$	102	140	0.83	Sl.S
1-heptanol	$CH_3(CH_2)_5CH_2OH$	116	176	0.82	0.09
1-octanol	$CH_3(CH_2)_6CH_2OH$	130	195	0.83	S
Miscellaneous Acids					
Propenoic Acid	CH_2=CH COOH	72	141	1.06	∞
Butenoic Acid	CH_3CH=CH COOH	86	189	1.02	8.3
Hydroxyethanoic Acid	CH_2OH COOH	76	d	-	S
2,3-dihydroxybutanedioic Acid	HOOC$(CHOH)_2$COOH	150	-	1.76	139
Sulfanilic Acid	$NH_2C_6H_4SO_3H.H_2O$	191	-	-	1.08
2-oxopropanoic Acid	CH_3CO COOH	88	165	1.27	∞
Methoxyethanoic Acid	CH_3O CH_2COOH	70	90	1.18	S
Ethanoic Anhydride		52	-	-	∞

Table V-1 (continued)

Organic Compound	Formula	Molecular Weight	Boiling Point (°C)	Specific Gravity	Solubility (gms/100 ml of water)
Aldehydes					
1-propanal	CH_3CH_2CHO	58	49	0.80	20.0
1-butanal	$CH_3(CH_2)_2CHO$	72	75	0.82	3.7
2-methyl propanal	$(CH_3)_2CH\ CHO$	72	62	0.79	11.0
1-heptanal	$CH_3(CH_2)_5CHO$	114	155	0.85	Sl.S
Glycols					
1,2-ethanediol	$CH_2OH\ CH_2OH$	62	197	1.12	∞
1,2-propanediol	$CH_3CH\ OH\ CH_2OH$	76	189	1.04	∞
Phenols					
Phenol	C_6H_5OH	94	182	1.07	6.7
Cresol	$CH_3C_6H_4OH$	108	202	1.04	2.4

Sl.S	Slightly Soluble
S	Soluble
d	decomposes
Subl	Sublimes

Table V-2. Commercial Pesticides

Commercial Pesticide	Names of Ingredients	Ingredient (Per cent)
Malthane (Trade Name)	DDD (Dichloro Diphenyldichloroethane)	6
	Xylene	84
	Malathion (o,o-Dimethyldithiophosphate of diethylmercaptosuccinate)	6
	Inert Ingredients	4
Ortho (Trade Name)	Malathion	29
	Benzene Hexachloride	10
	Solvents	43
	Inert Ingredients	18
DDT in Solvent	DDT	25
	Xylene	67
	Emulsifier	8

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